# FABP4 REGULATION OF UCP2 EXPRESSION IN INFLAMMATORY AND REDOX SIGNALING IN ADIPOSE TISSUE MACROPAHGES

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DEDICATION

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For my family, you have had my back since day one and have always been the first people I call to share my triumphs and defeats. You have fostered my growth, taught me to be independent and honest and always believed in me more than I could for myself. I am fully aware of how immensely fortunate I am to have you, and I hope I made you proud.

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ABSTRACT

Obesity has become a growing epidemic that has greatly increased the prevalence of a variety of metabolic syndromes, such as type II diabetes, cardio vascular diseases, and non-alcoholic fatty liver disease. A major factor that links adiposity to systemic metabolic dysfunction is the resident immune cells in the adipose tissue that exhibit enhanced inflammatory and oxidative stress characteristics. In order to uncouple obesity from these diseases, it is paramount to understand the molecular events that control the inflammation associated with the adipose tissue. The expression of the fatty acid-binding protein 4 (FABP4) in macrophages has demonstrated to be a key determinant of inflammation and oxidative stress where ablation of macrophage FABP4 is protective against obesity related disorders. Furthermore, this decrease in macrophage inflammation was shown to be dependent on the upregulation of uncoupling protein-2 (UCP2) and sirtuin-3 (Sirt3). Collectively these proteins maintained mitochondrial and endoplasmic reticulum homeostasis through redox balance and increased levels of intracellular monounsaturated fatty acids. This further lowered the level of reactive oxygen species, cysteine oxidation and inflammatory cytokine production, all of which have been linked to metabolic dysfunction. The work herein provides greater mechanistic insight into the induction of low-grade, chronic inflammation observed with obesity, which ultimately provides exciting potential for new therapeutic targets.

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# CHAPTER ONE

## Obesity induced inflammation and macrophage polarization

Written by Kaylee Steen

## FINANCIAL AND HEALTH BURDEN OF OBESITY

Through genetic factors, increased prevalence of the western-style diet and a sedentary lifestyle, obesity has become an annual $147 billion burden on the United State's health care system (data based on 2016 CDC statistics). While obesity is typically preventable with proper diet and exercise, for the 200 billion overweight individuals around the globe, prevention is only part of the solution (1). In order to uncouple the health care costs from the obese state, the mechanisms leading to obesity-related diseases need to be determined. This includes aberrant cellular signaling events associated with the adipose tissue, as this is the epicenter in which systemic disease results.

From a whole body perspective, a high caloric diet increases blood glucose concentration, which in turn triggers pancreatic secretion of insulin where it binds to the insulin receptor expressed on myocytes and adipocytes. Upon ligand binding, the insulin-signaling cascade activates the translocation of Glut4 to the plasma membrane and subsequent glucose uptake in adipocytes. While this signaling process maintains blood glucose homeostasis, it can also lead to nutrient overload in the tissue as excess glucose is converted to lipids through citrate metabolism. These lipids are stored in the form of triglycerides, which can be hydrolyzed to free fatty acids in times of nutrient deprivation in a process called lipolysis. However, as an adipocyte begins to expand from continual lipid synthesis, the cell is exposed to a variety of stress conditions, including hypoxia and apoptosis (2, 3). This cellular stress leads to insulin resistance, and once the adipocyte can no longer sense insulin, lipolysis will be activated and free fatty

acids are released even under nutrient rich conditions. Circulating fatty acids can accumulate in other organs as well as initiate a variety of systemic signaling cascades, collectively termed lipotoxicity (4).

## IMMUNITY AND THE ADIPOSE TISSUE

Inflammatory signaling is one such signaling event that is enhanced in obese adipose tissue, as a wide range of immune cells are recruited to the tissue by free fatty acids and cytokines, such as monocyte chemotactic protein-1 (MCP-1) and leukotrienes (Figure 1). The presence of these inflammatory immune cells include, but are not limited to, T cells, neutrophils, eosinophils and macrophages, all of which have demonstrated to regulate the metabolic homeostasis of the adipose tissue (5-9). Depending on the physiological state of the adipocytes and the biomolecules secreted from them, these immune cells can either be anti-inflammatory or proinflammatory, which greatly contributes to the metabolic fate of the adipose tissue (10).

These secretory factors are released by the adipocytes (i.e. fatty acids or cytokines) as well as resident immune cells that are already present in the tissue. In either case, cells respond to the local stress environment of the tissue and initiate signaling cascades. It is the regulation of these signaling events that can be the tipping point in metabolic health or disease, within the context of obesity (11, 12). Interestingly, it is estimated that around one-third of the obese population maintains metabolic health, and it is believed to be due to a reduced

inflammatory state in these individuals, particularly in relation to the macrophage population. It is therefore conceivable that by determining the inflammatory pathways that are activated in adipose tissue macrophages, the underlying causes of obesity-related diseases can be prevented (Figure 1).

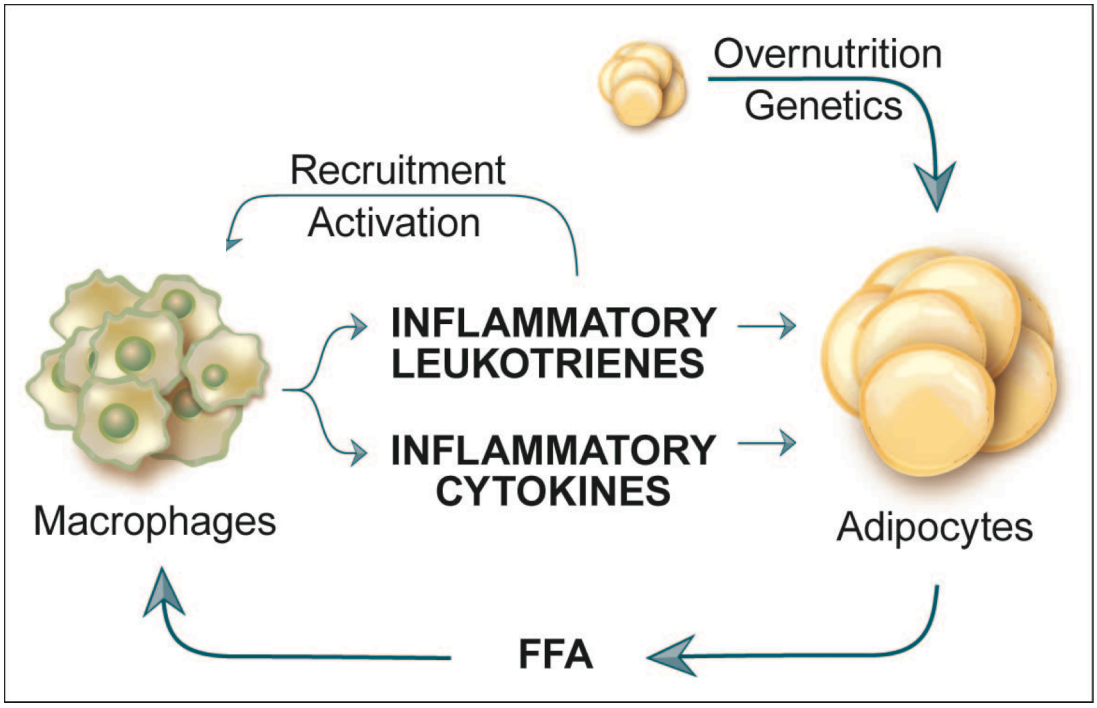


**Figure 1. Adipocyte and macrophage signaling in obese adipose tissue.** Expanding adipocytes secrete free fatty acids (FFAs) as well as other chemoattractants recruit macrophages to the adipose tissue and polarize the cells to an inflammatory state. The proinflammatory cytokines secreted by the macrophage are sensed by the adipocyte and can lead to insulin resistance (image adapted from A. Hertzel).

Macrophages are innate immune cells that are most commonly known for their ability to phagocytose pathogens and present the resulting antigen to T cells. What is less appreciated is the wide variety of macrophage phenotypes that exist, which allow the cells to fine tune their activity based on physiological need. Encompassing all these different characteristics, macrophages can be broadly classified into M1 (classically activated) and M2 (alternatively activated) states with opposing inflammatory phenotypes (13).

M1 macrophages exhibit inflammatory characteristics through elevated levels of reactive oxygen species (ROS) and cytokines, such as interleukin-1 beta (IL-1$\beta$) and tumor necrosis factor-alpha (TNF$\alpha$). The production of these and other inflammatory factors have all been strongly linked to insulin resistance and type II diabetes (14-16). On the other hand, M2 macrophages are associated with lean and, or metabolically healthy adipose tissue. In fact, M2 macrophages have been shown to promote metabolic homeostasis by producing anti-inflammatory factors, such as catecholamines and IL-10 that maintain insulin sensitivity (17). What is particularly incredible about macrophages is the plasticity in which they respond to their local environment by polarizing between an M1 and M2 state. These observations were supported using *in vitro* experiments that demonstrated gene expression profiles of cultured 3T3-L1 adipocytes and macrophages were altered when treated with conditioned media of the other cell type, respectively (18). This further demonstrates the potential consequences obesity can have on cellular function (19, 20).

An efficient mechanism that enables macrophages to quickly respond to local signals is through modulation of their metabolism. An M2 macrophage is typically longer lived than M1 macrophages, and therefore utilizes oxidative phosphorylation and fatty acid oxidation for its primary fuel sources. This is associated with a tightly packed and order cristae of the mitochondria that prevent electrons from prematurely moving into the matrix and reacting with oxygen. However, when a macrophage receives an inflammatory stimulus, the cristae become dispersed and the electron transport chain complexes become disorganized. This not only increases aerobic glycolysis and lactate production, but it also elevates the production of ROS (19, 21).

Another outcome of metabolic reprograming is the accumulation of intermediate metabolites. For example, succinate is a TCA cycle metabolite that accumulates under inflammatory stimuli and inhibits the pryolyl hydroxylases thereby stabilizing the hypoxic inducible factor-1 alpha (HIF-1$\alpha$) (22). This transcription factor upregulates inflammatory cytokines (i.e. IL-1$\beta$) and enzymes involved in glycolysis (further enhancing aerobic glycolysis). Another essential metabolite that accumulates during inflammatory metabolic reprogramming is itaconate. This molecule is synthesized by immune responsive gene 1 using cis-aconitate as its substrate and has been shown to prevent mitochondrial substrate level phosphorylation. However, itaconate is also an essential resolving agent of macrophage inflammation as knockout of Irg1 exhibited an increase in IL-1$\beta$ and IL-18 expression (22, 23). These data collectively highlight the essentiality of a

dynamic metabolic network in order for macrophages to respond to a physiological or pathological state.

## FABP4 EXPRESSION AND MACROPHAGE POLARIZATION

Of the many molecular events that lead to macrophage polarization, the expression of the fatty acid-bind 4 (FABP4) has demonstrated to play a critical role. This 15 kDa cytoplasmic protein binds to non-esterified long chain fatty acids in a one-to-one stoichiometry in order to shuttle the fatty acids inter- and intracellularly (24). The importance of FABP4 in metabolic homeostasis was first determined over twenty years ago by studying the whole body FABP4 knockout (AKO) in C57BL/6 mice. When put on a high fat diet, the AKO mice became as obese as wild type mice, but they did not develop insulin resistance (25, 26). Furthermore, when crossed with the atherosclerosis Apo/E mouse model or when given an allergen challenge, AKO mice continued to exhibit protection against these inflammatory-linked diseases (27, 28). It is now suggested this phenotype is due to the adipose tissue macrophage profile in that these macrophages exhibit an M2-like polarization (29). Due to the vast clinical implications these observations have, the last twenty years have been dedicated to uncovering the mechanistic role of FABP4 signaling in macrophages.

## MACROPHAGE INFLAMMATION AND OXIDATIVE STRESS

Inflammatory macrophages play a critical role in producing cytokines and reactive oxygen species. Both molecules are essential in propagating signaling events, and the two have intersecting yet distinct functions. Of the pathways that upregulate cytokine expression, the nuclear factor kappa B (NF-κB) is one of the most central pathways. NF-κB is basally localized to the cytoplasm through complex formation with IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), which masks the NF-κB nuclear localization signal. NF-κB can be relived of this inhibition through activation of toll-like receptor 4 (TLR4) by ligands, such as lipopolysaccharide (LPS) and saturated fatty acids. Once the inflammatory signal is received, IκB kinase (IKK) will phosphorylate and promote the degradation of IκBα, allowing NF-κB to translocate to the nucleus and upregulate inflammatory associated genes. Many of these cytokines, such as TNFα, are secreted and signal to local adipocytes, which further activates the NF-κB pathway in these cells (30-32).

One consequence of activating the NF-κB pathway in adipocytes is the expression of stress kinases, such as JNK (c-Jun N-terminal kinase) and ERK (extracellular signal-regulated kinase). The upregulation of JNK and ERK have strong implications in the development of insulin resistance through serine phosphorylation of the insulin receptor substrate 1 (IRS1). IKK has also been shown to phosphorylate IRS-1 at serine residues, which ultimately signals for the

degradation of IRS-1 and prevents insulin-dependent glucose uptake in adipocytes (12, 33, 34).

In macrophages, NF-κB also targets the proteins that form the inflammasome complex, which is a major inflammatory signaling node in immune cells. The inflammasome proteins include NLRP3 (NACHT, LRR and PYD domains-containing protein 3), pro-caspase-1, ASC and pro-IL-1β. Signals, such as LPS, are considered to be priming events that increase inflammasome protein concentrations in the cytoplasm. Once this occurs, an activation signal is required, such as ATP, ROS, β-amyloids and other factors (35, 36). Whether these factors act as direct or indirect ligands for the NLRP3 receptor, the downstream event that follows is the self-cleavage of pro-caspase-1, which in turn cleaves pro-IL-1β. Secretion of IL-β leads to systemic effects, such as beta-cell exhaustion of the pancreas that inhibits insulin release (14, 16).

Reactive oxygen species is another hallmark of inflammatory macrophages. There are multiple locations where ROS is produced, and the local accumulation of ROS has different consequences. For instance, ROS produced by NADPH oxidase occurs in the cytoplasm and facilitates elimination of pathogens. This is a major source of the antimicrobial capability of macrophages in which both ROS and reactive nitrogen species (RNS) are synthesized. Another source of ROS production is in the mitochondria that results from both physiological and abnormal oxidative phosphorylation (37).

Under physiological conditions, the mitochondria will produce a basal level of superoxide anion due to unpaired electrons entering the mitochondrial matrix and reacting with oxygen. This is quickly metabolized to hydrogen peroxide by superoxide dismutase and further converted to water by antioxidants, such as peroxiredoxin 3 (38, 39). However, when a macrophage receives an inflammatory signal, the dynamics of the electron transport changes such that elevated levels of superoxide anion is produced. This ultimately leads to an increase in total ROS levels, with hydrogen peroxide being of particular importance. Hydrogen peroxide can initiate signaling events though oxidation of protein cysteine residues (40). Furthermore, hydrogen peroxide has a longer half-life compared to the other ROS members and can therefore diffuse out of the mitochondria and oxidize cytoplasmic proteins. This oxidation event constitutes one mechanism in which cells propagate signal transductions. In fact, cellular processes, such as cell differentiation and insulin secretion require acute bursts of ROS (40, 41). However, a sustained and elevated production of ROS can lead to cellular dysfunction through protein unfolding, apoptosis, reactive lipid aldehyde production as well as sustained activation of the NF-κB and inflammasome pathways (29, 40, 42-45).

## REACTIVE OXYGEN SPECIES AND CELLULAR SIGNALING

Between the physiological and pathological roles of ROS, hydrogen peroxide is a major contributor to both, primarily through the oxidation of cysteine residues. While cysteine residues do not represent a large percentage of the amino acid

sequence for a protein, it is overrepresented in catalytic sites and is highly conserved among species. Furthermore, due to disulfide bond formation, cysteine residues are of structural significance (40, 46). Collectively, these oxidation events can rapidly alter protein-protein interaction, enzymatic activity, cellular localization, and protein stability. However, in order for redox signaling to have physiological significance, the oxidation of cysteine thiols to sulfenic acids or disulfide bonds must be a reversible process carried out by several types of antioxidants. On the other hand, pathological oxidation results from sustained levels of hydrogen peroxide, where sulfenic acids are further converted to sulfinic and sulfonic acids. These levels of oxidation are irreversible, and it typically renders the protein inactive or leads to unfolding and aggregation (Figure 2) (47, 48).

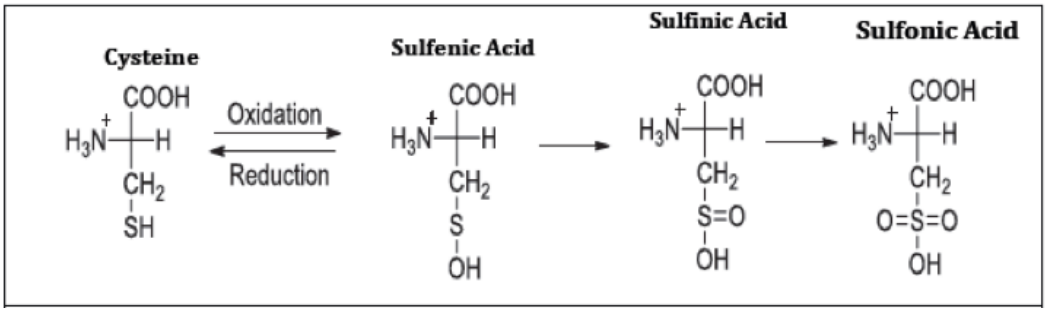


**Figure 2. Levels of cysteine oxidation**. The thiol on a cysteine residue is reversibly oxidized to sulfenic acid, and can be reduced by several types of antioxidants. However, further oxidation to sulfinic and sulfonic acid are irreversible and typically render the protein inactive, as the chemical nature of the protein will change as a result of these oxidative modifications.

In an attempt the maintain protein homeostasis during oxidative stress, the cell will initiate the unfolded protein response (UPR) in both the endoplasmic reticulum (ER) and the mitochondria. Collectively, mitochondrial dysfunction and ER stress have been implicated in a variety of metabolic syndromes (49-53). The ER is the major site for protein folding, and through favorable thermodynamics and chaperone assisted folding, intramolecular disulfide bonds are formed, reduced and reformed in order to obtain the correct tertiary structure. Under heightened oxidative stress, this process is perturbed, and a variety of chaperones, kinases and transcription factors are activated to reestablish protein homeostasis. During the initial stages of protein unfolding HSP 70 chaperone, BiP (binding immunoglobulin protein) and the NF-κB pathway are activated to prevent apoptosis. However, if the capacity of BiP and other chaperones are overwhelmed, stress kinases, such as IRE1 and proapoptotic transcription factors, such as C/EBP homologous protein (CHOP) are activated in order to protect the rest of the tissue (54-56).

Similar pathways are also activated in the mitochondria, as proteins will unfold as a result of mitochondrial derived ROS. Under these conditions, oxidative modifications change the chemistry of protein structure, and hydrophobic residues are exposed during the conformational changes that occur as a result of oxidation. As a counteractive mechanism, the mitochondria upregulates heat shock protein chaperones, such as HSP 60, to facilitate protein refolding. However, with sustained levels of ROS, proteases including Clp protease and

Lon protease are activated to prevent aggregation of these hydrophobic patches (43, 57). Finally, just like the ER unfolded protein response, the mitochondria will activate apoptotic pathways if homeostasis cannot be achieved. Importantly, one such consequence of the mitochondrial intrinsic apoptotic pathway is activation of the inflammasome, which is a central player of macrophage polarization described in this thesis (36, 42, 58).

Due to this intimate relationship between cellular function and protein homeostasis, maintaining the cellular redox capacity is of the utmost important. Antioxidants are probably one of the most widely appreciated systems that reduce the level of ROS and prevent higher oxidation of cysteine residues (59-61). These antioxidants include a diverse range of enzymes (including multiple isoforms), small peptides (i.e. glutathione), vitamins and other molecules. These factors may directly scavenge free radicals, as is the case with glutathione, or they can initiate upstream events that prevent the initial production of ROS. The uncoupling protein 2 (UCP2) performs the latter in mitochondrial derived ROS.

UCP2 is a transmembrane protein that lowers the mitochondrial membrane potential by transporting 4-carbon molecules into the cytoplasm (62). Lowering the membrane potential facilitates efficient electron movement across the electron transport chain complexes, thereby reducing the chance of an electron reacting with oxygen (63). Lowered expression of UCP2 has demonstrated to have essential regulatory functions of pathogen elimination in macrophages.

However, in the context of obesity-related metabolic disease, increased expression of UCP2 is correlated with a lower risk of type-II diabetes and overall improved metabolic health (64, 65). These findings make UCP2 an attractive protein to study as a potential therapeutic in chronic inflammation by modulating redox homeostasis. Of particular importance, Xu et al. demonstrated the expression of UCP2 was negatively regulated by FABP4 in macrophages. This provides a critical link between FABP4 and redox signaling in the regulation of adipose tissue macrophage polarization.

## GOALS AND OBJECTIVES

The link between obesity and metabolic health is strongly connected to both inflammation and oxidative stress events. This is supported by observations that immune cell populations differ in lean and obese adipose tissue as well as in obese metabolically healthy and unhealthy individuals. Furthermore, in several mouse studies, metabolic health was maintained during a high fat diet when inflammation was blunted (17, 25, 66-68). One explanation for these results is that inflammatory factors and ROS signal adipocytes to upregulate stress kinases. This in turn leads to insulin resistance and improper lipid storage (33, 68, 69). However, in order to fully understand the disease progression associated with obesity, the pathways that are altered in a polarizing macrophage requires further study. This thesis is a culmination of work that has been conducted related to FABP4 and UCP2 expression in macrophages that has dramatic effects in regulating macrophage inflammatory and oxidative stress pathways. As

a result of the work described herein, the molecular mechanisms in which a macrophage maintains an anti-inflammatory characteristic, even under obese conditions, has been more clearly elucidated.

The first objective in this thesis was to define the mechanism in which FABP4 null macrophages exhibited an M2-like phenotype. As discussed in chapter two, it was determined to be largely due to the upregulation of the uncoupling protein 2 (UCP2) in response to increased levels of palmitoleic acid. UCP2 expression reduced reactive oxygen species, inflammatory cytokines and endoplasmic reticulum stress and improved mitochondrial function. However, UCP2 upregulation was not able to explain the increased beta-oxidation of FABP4 null macrophages. This phenotype was determined to be dependent on the induction of sirtuin 3 (SIRT3). Moreover, SIRT3 expression also demonstrated to play a role in blunting the expression of inflammatory cytokines and ROS production in addition to the effects of UCP2.

Although these data helped elucidate the proteins responsible for reduced inflammation and oxidative stress, there were still mechanistic gaps as to how these pathways were altered. This led to the third objective in examining the role ROS plays in modifying cysteine residues through oxidation events. As shown in chapter 4, FABP4 null mice had enhanced antioxidant expression and concomitant reduced global cysteine oxidation, mitochondrial unfolded protein response and inflammasome activation. Another mechanism in which

macrophages alter their inflammatory phenotype is through changes in metabolic dynamics. In the final objective of this thesis, it was observed that the tricarboxylic acid (TCA) cycle was altered, leading to elevated levels of itaconate. These data indicate that ROS signaling and metabolic alterations are dependent on the expression of FABP4 in macrophages in determining the inflammatory fate of the cell.

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# CHAPTER TWO

## Uncoupling Lipid Metabolism from Inflammation Through FABP-dependent Expression of UCP2

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This chapter contains an original research article previously published. Kaylee Steen generated the R126Q cell line used in figure 1 and helped in the editing process of this manuscript
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**SUMMARY**

Chronic inflammation in obese adipose tissue is linked to endoplasmic reticulum (ER) stress and systemic insulin resistance. Targeted deletion of the murine adipocyte fatty acid binding protein (FABP4/aP2) uncouples obesity from inflammation, although the mechanism underlying this finding has remained enigmatic. Herein we show that inhibition or deletion of FABP4/aP2 in macrophages results in increased intracellular free fatty acids and elevated expression of uncoupling protein 2 (UCP2) without concomitant increases in UCP1 or UCP3. Silencing of UCP2 mRNA in FABP4/aP2 deficient macrophages negated the protective effect of FABP loss and increased ER stress in response to palmitate or lipopolysaccharide (LPS). Pharmacologic inhibition of FABP4/aP2 with the FABP inhibitor HTS01037 also up-regulated UCP2 and reduced expression of BiP, CHOP and XBP-1s. Expression of native FABP4/aP2 (but not the non-fatty acid binding mutant, R126Q) into FABP4/aP2 null cells reduced UCP2 expression suggesting that the FABP-FFA equilibrium controls uncoupling protein 2 expression. FABP4/aP2 deficient macrophages are resistant to LPS-induced mitochondrial dysfunction, exhibit decreased mitochondrial protein carbonylation and UCP2-dependent reduction in intracellular reactive oxygen species. These data demonstrate that FABP4/aP2 directly regulates intracellular FFA levels and indirectly controls macrophage inflammation, and ER stress by regulating the expression of UCP2.

## INTRODUCTION

Obesity-linked metabolic disorders including insulin resistance, fatty liver disease, and coronary arterial disease share the common signature of chronic inflammation and endoplasmic reticulum (ER) stress (1, 2). Macrophage and T cell infiltration and activation in adipose tissue plays a key role in affecting adipokine synthesis and secretion thereby regulating systemic insulin resistance (3). Inflammatory cytokines increase oxidative stress and decrease the protein-folding efficiency of the ER initiating a counter regulatory unfolded protein response (UPR) (4) involving pancreatic ER kinase (PERK), activating transcription factor-6 (ATF6), and inositol requiring enzyme 1 (IRE1). Such concomitant activation leads to the downstream activation of response pathways and the induction of inflammatory signaling networks via JNK (c-Jun N-terminal kinase) and/or NF-κB (nuclear factor kappa B) (1).

Lipid metabolism in macrophages has been shown to play an important role in triggering inflammation and ER stress (5, 6) and has led to the identification of critical proteins that regulate the obesity-metabolic disease axis. For example, genetic ablation of the adipocyte fatty acid binding protein (FABP4, also known as aP2) in macrophages alone is sufficient to protect the mice from development of atherosclerosis and dyslipidemia (7). FABP4/aP2 is a cytoplasmic fatty acid carrier protein that mediates intracellular fatty acid trafficking and a number of hypotheses have been proposed for why the loss of FABP4/aP2 results in metabolic improvement (6). Moreover, small molecules that target FABP4/aP2

have been developed as potential therapeutics (8). However, conflicting reports exist concerning the effectiveness of these inhibitors using cell-based and animal models (9). FABP4/aP2 deficient macrophages exhibit suppressed inflammatory signaling, attenuated activation of the NF-κB pathway and decreased ER stress (6, 10). Consistent with a role for FABP4/aP2 as a key determinant in obesity-linked inflammation, genetic variation in the human FABP4/aP2 promoter that leads to decreased expression of the protein in adipose tissue is associated with lower serum triglyceride levels, reduced coronary disease and type 2 diabetes (11). The biochemical processes underlying the effects of FABP4/aP2 deficiency on macrophage lipid metabolism and ER stress and inflammatory pathways are not understood, but may be linked to the accumulation of intracellular unsaturated fatty acids, particularly palmitoleic acid (6, 10).

The investigation herein describes the novel finding that UCP2 is up-regulated selectively in macrophages from FABP4/aP2 null mice and that increased expression of UCP2 plays an important and essential role in alleviating ER stress and decreasing inflammation (12, 13). Unlike its structural homolog UCP1 that is highly expressed in brown fat, UCP2 is more broadly expressed in various tissues and cells, functions as a sensor of mitochondrial oxidative stress and is generally considered to be cytoprotective (14). Moreover, unsaturated fatty acids increase UCP2 expression in macrophage cells suggesting that the FABP – fatty acid equilibrium is central to mediating metabolic homeostasis.

## MATERIALS AND METHODS

**Cell lines.** FABP4/aP2 knockout and wild type macrophage cells were maintained in RPMI 1640 (Invitrogen) with 5% fetal bovine serum (FBS). Raw264.7 macrophages as well as UCP2 knockdown Raw264.7 macrophages were maintained in DMEM (Invitrogen) with 10% FBS. Peritoneal macrophages were isolated from C57Bl/6J animals. 1-2 $X10^6$ cells were plated and incubated overnight (15).

**Intracellular fatty acid analysis.** Monolayers of cells were washed with phosphate-buffered saline and harvested into 2 mL of 100 mM sodium acetate (pH 3.9). Lipids were extracted into hexane: isopropanol: $H_2O$ (3:2:2) and centrifuged at 3000 rpm for 10 minutes to achieve phase separation. The aqueous phase was dried under nitrogen and lipids solubilized in 1 mL chloroform. Samples were loaded onto equilibrated HF Bond Elut NH2 column (Agilent Technology), washed with chloroform: isopropanol (2:1) to remove neutral lipids and fatty acids eluted with 2% acetic acid in diethyl ether. The fatty acid eluate was dried and resolubilized in isopropanol for measurement of fatty acid abundance (NEFA kit, Wako) or submitted to the Metabolomics Resources Core of the Mayo Clinic (Rochester MN) for fatty acid composition analysis. The fatty acid composition analysis was carried out by LC-MS with C17:0 spiked in each sample as an internal standard.

**Isolation of stromal vascular cells.** Epididymal fat pads were dissected from wild type C57Bl/6J and FABP4/aP2 KO mice (n=6) maintained on high fat diet for

12 weeks (16). Briefly, fat pads were minced and digested with Type I collagenase in Krebs-Ringers-Hepes buffer supplemented with 10 mg/ml BSA. After incubation at 37° C for 1 hour, the mixture was filtered with cell strainer (100μm Nylon, FALCON) to remove undigested tissues. The stromal vascular fraction was collected by centrifugation at 500 g for 10 minutes. The stromal vascular fraction was washed and TRIzol reagent used for RNA isolation. All experimental procedures using animals were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee.

**shRNA knockdown of UCP2 in macrophages.** Raw264.7 and FABP4/aP2 KO macrophages were transduced with shRNA lentivirus as described previously (17). GFP scrambled and Ucp2 targeting sequences were obtained from Open Biosystems. Ucp2 (NM_011671) targeting sequence (UCP2 kd): 5'CCGGTCTCCCAATGTTGCCCGTAATCTCGAGATTACGGGCAA CATTGGGAGATTTTTG-3'; alternative UCP2 targeting sequence (UCP2-2 kd): CCGGCCCA GCCTACAGATGTGGTAACTCGAGTTACCACATCTGTAGGCTGGGTTTTTG-3'; the scrambled sequence: 5'-AACGTACGCGGAATACTTCGA-3'.

**Expression analysis by Quantitative Real time PCR (qRT-PCR).** Total RNA was isolated using TRIzol reagent (Invitrogen), and reverse transcribed to cDNA using iScript according to the manufacturer's protocol (Bio-Rad). qRT-PCR amplification was performed on a Bio-Rad CFX 96 Real-Time System using

SYBR Green Supermix (Bio-Rad). Transcription factor II E (TFIIE) was used as an internal control to normalize expression unless specified otherwise. Primer sequences are provided in Table 1.

**Reverse transcription PCR analysis (RT-PCR)**. RT-PCR was used to identify UCP1, UCP2, and UCP3 expression in macrophages. Primers for UCP1 forward: GCCAGGCTTCCAGTACATTA, UCP1 reverse: TGGTACGCTTGGGTACTGTCC; UCP2 forward: CCAGAGCACTGTCGAAGCCT, UCP2 reverse: GCAGCCATTAGGGCTCTTTTG; UCP3 forward: AGAACCCAGGGGCTCAGAG, UCP3 reverse: AAAACGGAGATTCCCGCAGTA.

**Cellular respiratory Assay.** Macrophage respiratory assay was performed on a XF24 extracellular flux analyzer (Seahorse Biosciences) (18). Macrophages were plated on V7 microplates at a density of 300,000 cells per plate, incubated overnight, and then cells were treated either with vehicle or lipopolysaccharide (LPS) (100ng/ml) for 6 hours. During the assay, cells were exposed to compounds in the following order: 2 μM oligomycin, 0.4 μM FCCP, and 4 μM antimycin A.

**Hydrogen Peroxide ($H_2O_2$) assay.** $H_2O_2$ quantification was determined using Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) according to the manufacturer's protocol with modification. Briefly, cells were scraped into

phosphate buffer (pH7.4) and inactivated at 95°C for 10 minutes. After spin down of cell debris, 50 µL of supernatant were loaded with 50 µL working solution. Following 30 minutes incubation, fluorescence was measured using a microplate reader with excitation at 540 nm and emission at 590 nm.

**Membrane potential measurement.** Mitochondrial proton motive force was measured by tetramethylrhodamine, methyl ester (TMRM) staining (Invitrogen). Briefly, cells were washed with PBS, and incubated in 1 mL of KRH buffer (pH7.4) with 20 nM final concentration of TMRM for 30 minutes. Then cells are washed with PBS and harvested into 300 µL KRH buffer. 150 µL of each sample was load into 96-well plate and fluorescence was measured using microplate reader with excitation at 531 nm and emission at 572 nm.

**Mitochondrial isolation.** Cells were scraped into isolation buffer (20 mM Tris (pH 7.4), 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.1 mM EGTA) supplemented with protease inhibitors and lysed with 20 strokes of a Dounce homogenizer. Homogenates were centrifuged at 700*g* for 10 minutes to remove nuclei and unbroken cells. Mitochondria were pelleted by centrifugation at 12,000*g for 15 minutes*.

**Immunoblotting.** Cells were lysed in RIPA buffer supplemented with protease inhibitors. Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membrane. After blocking, membranes were incubated with

primary antibody overnight at 4° C. Membranes were washed and incubated with secondary antibody conjugated to LI-COR IR Dye for 1 hour and visualized using LI-COR Odyssey infrared imaging (LI-COR biotechnologies). The antibodies used were anti-UCP2 (Santa Cruz Biotechnology, C-20), anti-HNE (Millipore), anti-DDIT3 (anti-CHOP) (Abcam), anti-GPR78 (Bip) (Santa Cruz Biotechnology, H-129), anti-Cox2 & anti-iNOS (BD Transduction Laboratories), anti-β-Actin (Sigma Aldrich) and anti-ATP synthase-α subunit (MitoSciences).

**Cytokine TNFα measurement.** Secreted TNFα in medium (8hr) was measured with Mouse TNF ELISA Set from BD Biosciences according to the manufacturer's instruction.

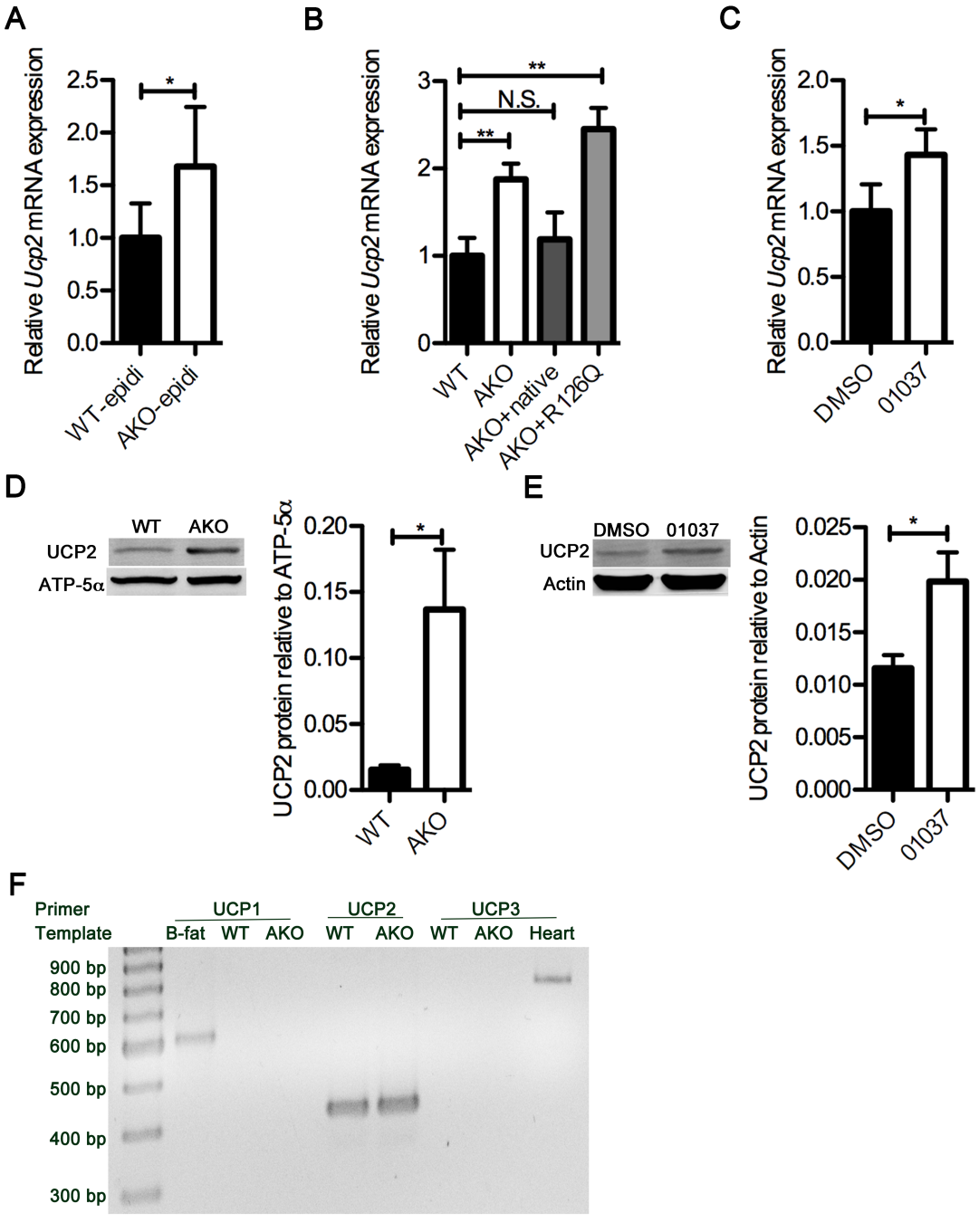
**Fatty acid oxidation assay.** Fatty acid oxidation was carried out as described by Wiczer and Bernlohr (19). Briefly, cells were incubated for 1 hour at 37°C in Krebs-Ringers-Hepes buffer (pH7.4), containing 5.4 mM glucose and 400 μM [$^{14}$C] palmitic acid bound to 100 μM fatty acid free BSA. Cells were scraped from the plates and transferred with media into 20 mL glass reaction vials containing a center reaction tube filled with 400 μL 1M sodium hydroxide. 70% perchloric acid was added to the media (final concentration of 7%), incubated for 1 hour with shaking at 80 rpm. After incubation, the content of the center tube was transferred into 10 mL liquid scintillation fluid and the $^{14}CO_2$ determined by liquid scintillation counting.

**Statistical analysis.** All data in the paper are expressed as standard deviation (± SD). Statistical significance was determined using an unpaired, two-tailed Student *t* test.

## RESULTS

**Loss or inhibition of FABP4/aP2 in macrophages leads to increased UCP2 expression.** Previous reports have demonstrated that the intracellular fatty acid pool in FABP4/aP2 deficient macrophages and adipocytes is increased and has an altered composition (6, 20). Work in other systems, particularly in the liver, has shown that the expression of UCP2 mRNA and protein is increased with obesity, regulated by fatty acids and that lipid overload leads to increased expression of UCP2 as part of a counter regulatory cycle (14, 21). Since FABPs establish an intracellular equilibrium between bound and free fatty acids, we hypothesized that loss of FABP4/aP2 would result in increased availability of lipid and up-regulation of UCP2. To test this hypothesis, we evaluated the expression of uncoupling proteins using qRT-PCR and determined that UCP2 mRNA is increased approximately 60% in FABP4/aP2 deficient SVF cells compared to that of wild type mice (Fig. 1A). Similarly, UCP2 mRNA levels were increased ~ 2-fold in cell lines derived from FABP4/aP2 deficient mice (referred to as AKO macrophages) compared to control cells (Fig. 1B). The phenotype of FABP4/aP2 deficiency in macrophages can be mimicked by treatment of cultured macrophages with a previously identified and characterized chemical inhibitor of FABPs, HTS01037 (8). Treatment of Raw264.7 macrophages with HTS01037

also significantly increases UCP2 mRNA level (Fig. 1C). To further confirm that the up-regulation of UCP2 is responsive to FABP4/aP2 deficiency, AFABP/aP2$^{-/-}$ macrophages were reconstituted with either a wild type FABP4/aP2 or a non-fatty acid binding mutant of AFABP/aP2 (R126Q) (22) to a level comparable to that of FABP4/aP2 in wild type macrophages (results not shown). Re-expression of wild type FABP4/aP2, but not the R126Q mutant, reduces UCP2 mRNA to a level comparable to that in wild type macrophages (Fig. 1B) implying that the intracellular FFA-FABP equilibrium is a major control element for UCP2 expression. Analysis of UCP2 protein levels in FABP4/aP2 deficient macrophages show that UCP2 is significantly increased compared to that of wild type (Fig. 1D). Moreover, the treatment of Raw264.7 macrophages with the FABP inhibitor HTS01037 also increases UCP2 protein level (Fig. 1E). Furthermore, the increased expression of uncoupling protein was specific for UCP2 as there was no evidence for any expression of UCP1 or UCP3 (Fig. 1F). These results in sum indicate that FABP4/aP2-FFA equilibrium controls the expression of the UCP2.


A
Relative Ucp2 mRNA expression
2.5
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AKO+R126Q
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Relative Ucp2 mRNA expression
2.0
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UCP2
ATP-5α
UCP2 protein relative to ATP-5α
0.20
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AKO
E
DMSO
01037
UCP2
Actin
UCP2 protein relative to Actin
0.025
0.020
0.015
0.010
0.005
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*
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01037
F
Primer
Template
UCP1
UCP2
UCP3
B-fat
WT
AKO
WT
AKO
WT
AKO
Heart
900 bp
800 bp
700 bp
600 bp
500 bp
400 bp
300 bp

**Figure 1**. **Loss of FABP4/aP2 increases UCP2 expression.** (A) UCP2 mRNA level normalized to macrophage F4/80 in the stromal vascular fraction of epididymal adipose tissue obtained from high fat diet fed wild type (WT) and AFABP/aP2$^{-/-}$ (AKO) mice. (B) UCP2 mRNA level in wild type, AKO, AKO+native (AFABP/aP2$^{-/-}$ macrophages reconstituted with WT AFABP/aP2), AKO+R126Q (AFABP/aP2$^{-/-}$ macrophages reconstituted with a non-fatty acid binding mutant of AFABP/aP2). (C) UCP2 mRNA level in Raw264.7 macrophages treated with 30 μM HTS01037 for 24 hours. (D) UCP2 protein expression in AKO and WT macrophage mitochondrial fraction determined by western blot. (E) UCP2 expression in Raw264.7 macrophages treated with 30 μM HTS01037 determined by western blot. (F) RT-PCR amplification of UCP1, 2 and 3 from wild type (WT) and FABP4/aP2$^{-/-}$ (AKO) macrophages. Amplification of UCP1 from brown fat (B-fat) was used as positive control for UCP1 expression, while UCP3 expression control was amplified from a heart cDNA sample. (* $p<0.05$, ** $p<0.01$)

**Unsaturated fatty acids induce UCP2 expression in macrophages via PPARγ.** Previous reports have shown that in multiple systems UCP2 expression can be induced by unsaturated fatty acids (14, 23-25). Moreover, FABPs are identified as lipid chaperones involved in establishment of the bound vs. free FFA equilibrium (16, 26). Consistent with previous work, intracellular free fatty acids in FABP4/aP2$^{-/-}$ macrophages were increased ~70% compared to that of wild type (Fig. 2A) (6). Moreover, fatty acid composition analysis revealed that monounsaturated fatty acid levels such as oleic acid (18:1), palmitoleic acid (16:1 *cis*) and palmitelaidic acid (16:1 *trans*) were selectively elevated in FABP4/aP2$^{-/-}$ macrophages (Fig. 2B). To test which molecular species of fatty acid could induce UCP2 expression in macrophages, Raw264.7 cells were treated for 24 hours with different fatty acids (4:1 FFA/bovine serum albumin) and the expression of UCP2 evaluated. The results demonstrate that polyunsaturated fatty acids, including DHA (22:6), EPA (20:5), linoleate (18:2) and the monounsaturated fatty acids oleate (18:1) and palmitoleate (16:1) can each induce UCP2 expression in macrophages, while the saturated fatty acid palmitate (16:0) was unable to elicit any response (Fig. 2C). Unsaturated fatty acids have been shown to act as ligands of a family of transcription factors - peroxisomal proliferator-activated receptors (PPARs) which are involved in regulating the expression of a cohort of genes involved in lipid metabolism (27). PPARγ is the major form of PPARs expressed in macrophages and suppresses the expression of a large set of inflammatory genes (28). qRT-PCR analysis shows that both PPARγ and its target genes such as liver X receptor alpha (LxRα), cluster of

differentiation 36 (CD36), arginase and stearoyl-CoA desaturase-1 (SCD1) were up-regulated in FABP4/aP2$^{-/-}$ macrophages compared to that of wild type macrophages. On the contrary, the expression of the proinflammatory gene, inducible nitric oxide synthase (iNOS), is down regulated in FABP4/aP2$^{-/-}$ macrophages (Fig. 2D). In addition, treatment of wild type peritoneal macrophages with the FABP inhibitor HTS01037 shows similar results (Fig. 2E). In order to determine the role of PPARγ in macrophage UCP2 expression, Raw264.7 cells were treated with the PPARγ agonist troglitazone. Troglitazone treatment increased the expression of UCP2 in macrophages, as well as LXRa (Fig. 2F). On the other hand, treatment of FABP4/aP2$^{-/-}$ macrophages with PPARγ antagonist GW9662 reduced UCP2 expression to a level comparable to that of wild type (Fig. 2G).

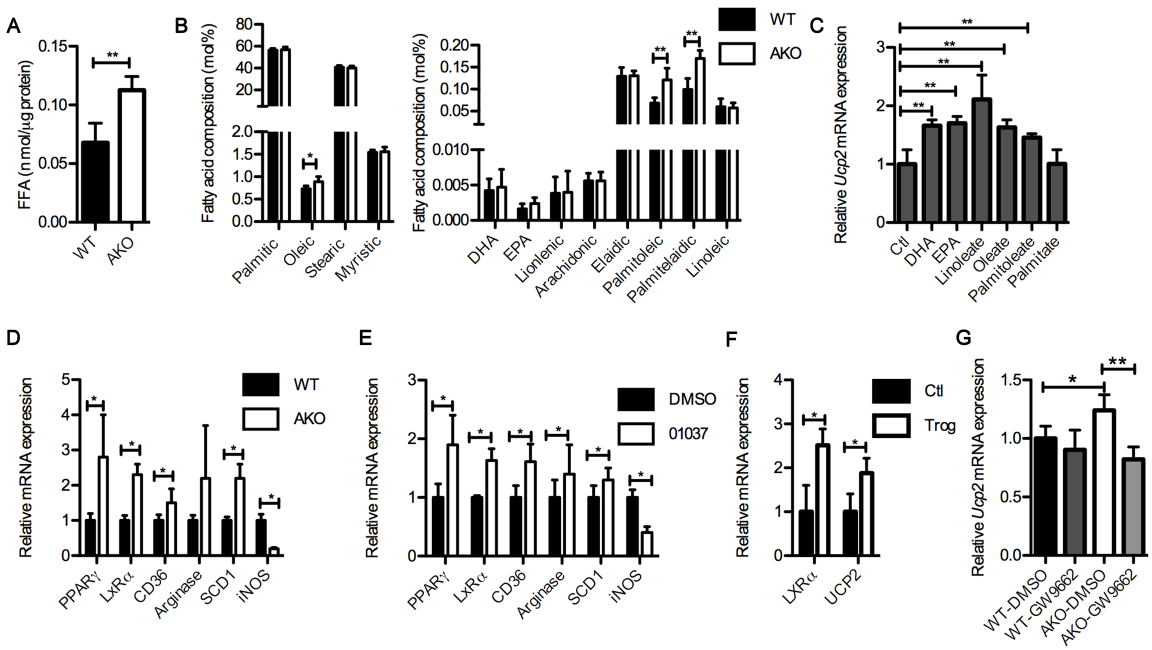



**Figure 2. Unsaturated fatty acids induce UCP2 expression via PPARγ.** (A) Intracellular free fatty acids measured in WT (wild type) and AKO (FABP4/aP2$^{-/-}$) macrophages. (B) Fatty acid composition in WT and AKO macrophages. (C) UCP2 mRNA level in Raw264.7 macrophages treated with 300 μM docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleate, oleate, palmitoleate, or palmitate. Fatty acids were added in complex to bovine serum album at a molar ratio of 4:1. (D) mRNA levels of PPARγ, liver X receptor alpha (LxRα), cluster of differentiation 36 (CD36), arginase, stearoyl-CoA desaturase 1 (SCD1) and inducible nitric oxide (iNOS) in WT and AKO peritoneal macrophages. (E) PPARγ, LxRα, CD36, Arginase, SCD1 and iNOS mRNA level in 10 μM HTS01037 treated WT peritoneal macrophages. (F) mRNA level of LxRα and UCP2 in Raw264.7 macrophages treated with 5 μM troglitazone for 24 hours. (G) mRNA level of UCP2 in WT and AKO macrophages treated with 5 μM GW9662 for 24 hours. (* $p<0.05$, ** $p<0.01$)

**UCP2 up-regulation negates palmitate-induced ER stress in FABP4/aP2 deficient macrophages.** The alleviation of lipid-induced macrophage ER stress can be accomplished either by knocking out FABP4/aP2 genetically or inhibiting the FABP-fatty acid interaction with small molecule inhibitors (6). In addition, the reduction in macrophage ER stress has been shown to offer protection against atherosclerosis (29). We therefore evaluated the role of UCP2 up-regulation in FABP4/aP2$^{-/-}$ macrophages as mediating reduced ER stress in palmitate-treated macrophages by silencing UCP2 in AKO and Raw264.7 macrophages (Fig. 3A and B). Spliced x-box binding protein 1 (XBP-1s) (30, 31) was markedly down-regulated in Raw264.7 cells treated with HTS01037, while the level of the unspliced form was increased, suggesting loss of FABP4/aP2 protects macrophages from ER stress (Fig. 4A). Treatment of UCP2 knockdown and control cells with HTS01037 shows that UCP2 silenced macrophages have a basal increase of XBP-1s and a blunted response to HTS01037 (Fig. 4B).

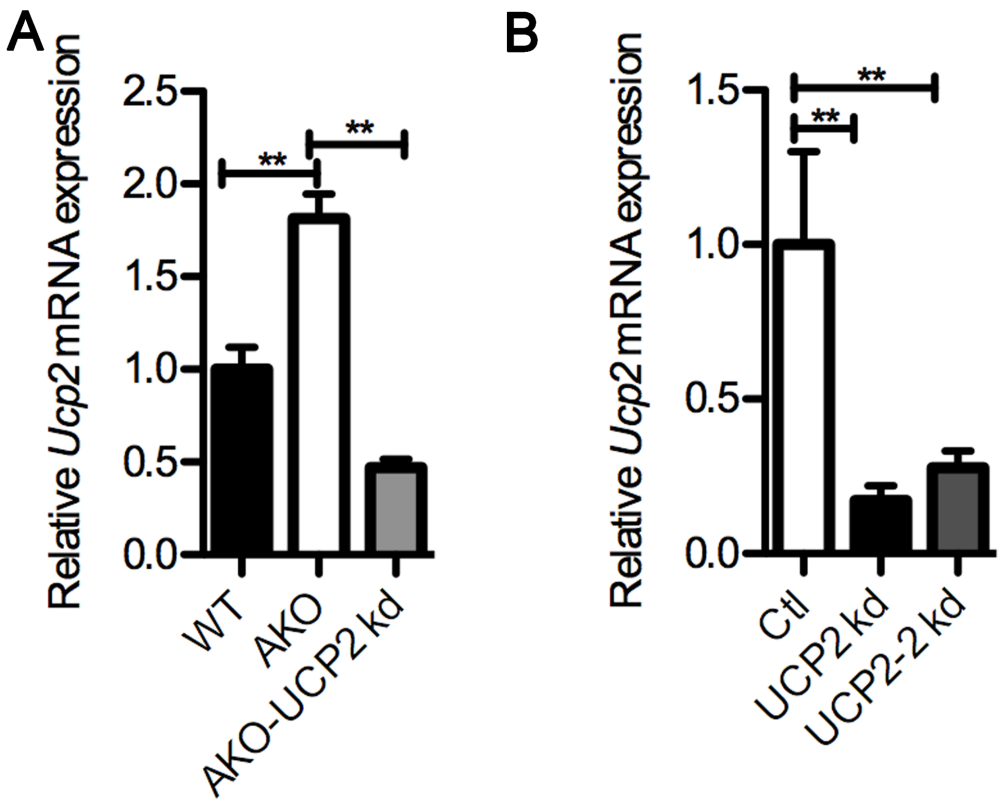
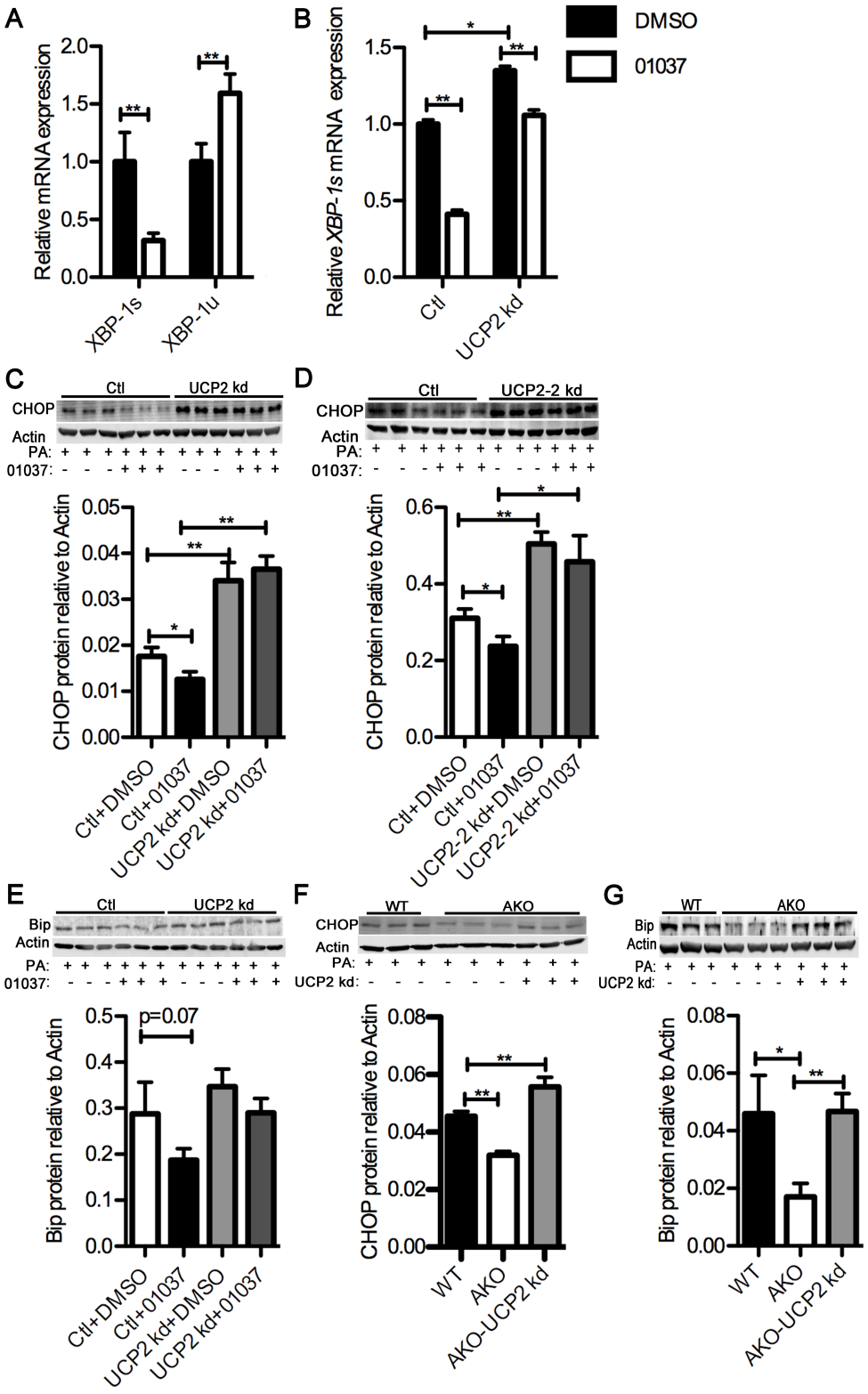



**Figure 3. Knock down of UCP2 in FABP4/aP2 deficient and Raw 264.7 macrophages.**

(A) UCP2 mRNA levels in wild type (WT), AKO, and AKO-UCP2 kd (UCP2 knockdown AKO macrophages). (B) UCP2 mRNA levels in control cells, UCP2 kd and UCP2-2 kd (alternatively silenced UCP2 knock down cells) Raw264 macrophages. (* $p<0.05$, ** $p<0.01$)

Lipid loading, especially palmitate treatment, has been shown to induce expression of proteins involved in ER stress response, such as C/EBP homologous protein (CHOP) and immunoglobulin heavy chain binding protein (Bip) (6). To assess the role of UCP2 induction in regulating ER stress response to palmitate, UCP2 knockdown and control cells were pretreated with HTS01037 or vehicle and palmitate-induced ER stress was evaluated. Expression of CHOP and Bip with palmitate treatment was markedly reduced in response to HTS01037 treatment in macrophages and this protection was negated in UCP2 knockdown macrophages (Fig. 4C-E). In order to further determine the role of UCP2 in inhibiting the ER stress response, UCP2 was knocked down in FABP4/aP2 deficient macrophages (AKO-UCP2 kd) and palmitate-induced ER stress evaluated. Importantly, palmitate treatment of macrophages showed increased expression of CHOP and Bip in wild type and AKO-UCP2 kd macrophages, as compared to FABP4/aP2$^{-/-}$ (AKO) macrophages (Fig. 4F and G). Taken together, these results strongly indicate that UCP2 expression in FABP4/aP2 deficient macrophages plays an important role in mediating the reduced lipid-induced ER stress.


A
Relative mRNA expression
XBP-1s
XBP-1u
B
DMSO
01037
Relative XBP-1s mRNA expression
Ctl
UCP2 kd
C
Ctl
UCP2 kd
CHOP
Actin
PA:
01037:
CHOP protein relative to Actin
Ctl+DMSO
Ctl+01037
UCP2 kd+DMSO
UCP2 kd+01037
D
Ctl
UCP2-2 kd
CHOP
Actin
PA:
01037:
CHOP protein relative to Actin
Ctl+DMSO
Ctl+01037
UCP2-2 kd+DMSO
UCP2-2 kd+01037
E
Ctl
UCP2 kd
Bip
Actin
PA:
01037:
p=0.07
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Ctl+01037
UCP2 kd+DMSO
UCP2 kd+01037
F
WT
AKO
CHOP
Actin
PA:
UCP2 kd:
CHOP protein relative to Actin
WT
AKO
AKO-UCP2 kd
G
WT
AKO
Bip
Actin
PA:
UCP2 kd:
Bip protein relative to Actin
WT
AKO
AKO-UCP2 kd

**Figure 4. UCP2 up-regulation mediates decreased ER stress in FABP4/aP2 deficient macrophages.**

(A) XBP-1s and XBP-1u mRNA levels in Raw264.7 macrophages treated with 30 μM HTS01037. (B) XBP-1s mRNA levels in UCP2 knockdown and control macrophage cell lines treated with 30 μM HTS01037 for 24 hours. (C-D) CHOP and (E) Bip abundance determined by western blot in UCP2 knockdown and control cells pretreated with vehicle or HTS01037 (30μM) for 3 hours and treated with 500 μM palmitate for 16 hours. (F) CHOP abundance determined by western blot in WT, AKO and AKO-UCP2 kd (UCP2 knockdown AKO macrophages) cells treated with 300 μM palmitate for 24 hours. (G) Bip abundance determined by western blot in WT, AKO and AKO-UCP2 kd cells treated with 500μM palmitate for 12 hours. (* $p<0.05$, ** $p<0.01$)

**UCP2 mediates the decreased inflammatory signaling in FABP4/aP2 deficient macrophages.** FABP4/aP2$^{-/-}$ macrophages have reduced expression of inflammatory proteins such as cyclooxygenase-2 (Cox2) and iNOS (7, 10). In order to determine if UCP2 is involved in the suppression of inflammatory signaling in FABP4/aP2 knockout macrophages, wild type and FABP4/aP2$^{-/-}$ macrophages were treated with lipopolysaccharide (LPS) in the presence or absence of genipin, an UCP2 inhibitor (32) and the inflammatory response was profiled. Consistent with previous studies, LPS-induced Cox2 expression and TNF-α secretion were significantly lower in FABP4/aP2$^{-/-}$ macrophages (Fig. 5A and B). Moreover, LPS and genipin co-treatment of FABP4/aP2$^{-/-}$ macrophages significantly increased Cox2 expression and TNF-α secretion in FABP4/aP2$^{-/-}$ macrophages compared to that of LPS treatment alone (Fig. 5A and B) suggesting that increased UCP2 expression may attenuate inflammatory responsiveness. To further confirm UCP2's role in suppressing inflammatory signaling, control cells and UCP2 knockdown Raw264.7 macrophages were pretreated with HTS01037 and stimulated with LPS or LPS+INF-γ (interferon gamma). HTS01037 treatment significantly reduced LPS induced Cox2 and LPS plus IFN-γ induced iNOS expression in control cells (Fig. 5C-E). However, the effect of HTS01037 was significantly reduced, if not totally abolished, in UCP2 knockdown macrophages (Fig. 5C-E). Taken together, these data indicate that UCP2 plays an important role in mediating the reduced inflammation in FABP4/aP2 deficient macrophages and that the key determinant of metabolic improvement in FABP deficient mice is up-regulation of UCP2.

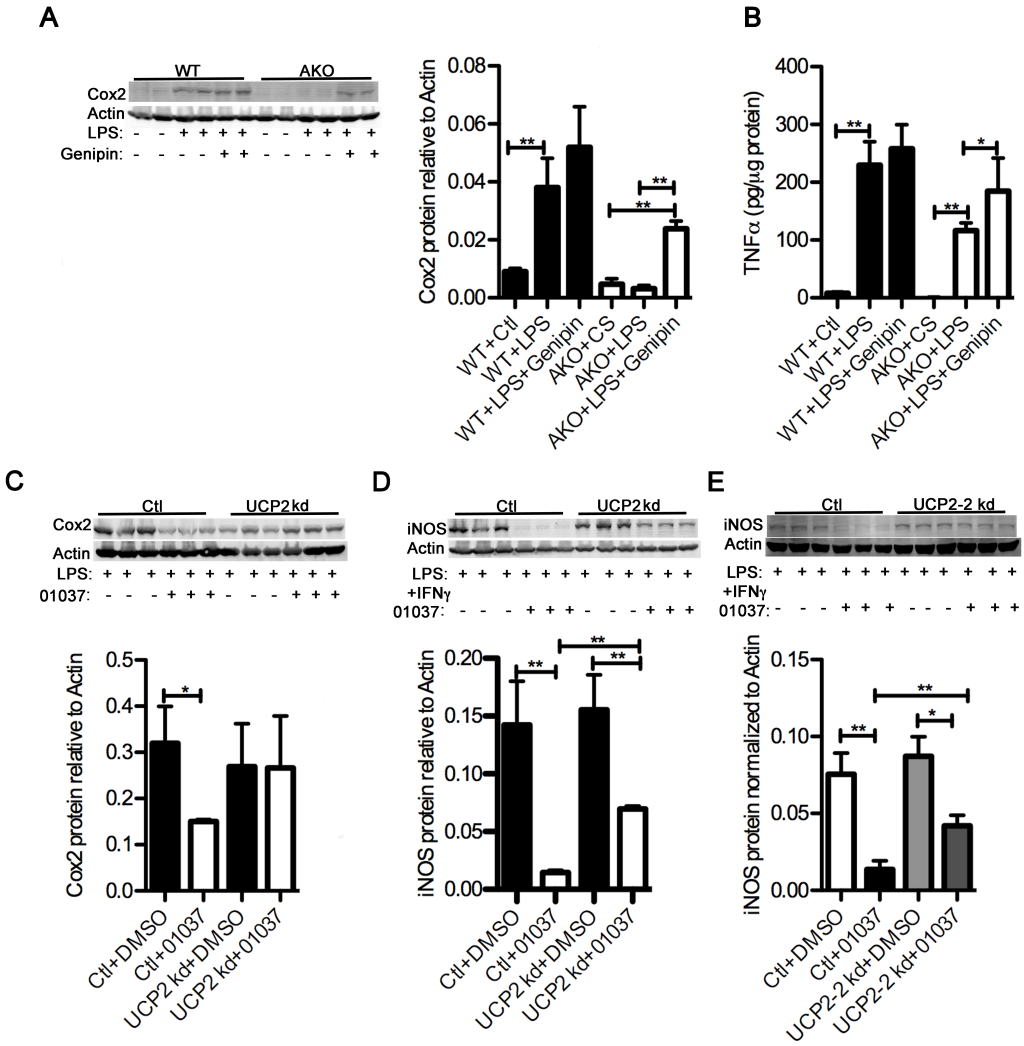
A
WT
AKO
Cox2
Actin
LPS:
Genipin:
Cox2 protein relative to Actin
0.08
0.06
0.04
0.02
0.00
WT+Ctl
WT+LPS
WT+LPS+Genipin
AKO+CS
AKO+LPS
AKO+LPS+Genipin
B
TNFα (pg/μg protein)
400
300
200
100
0
C
Ctl
UCP2kd
Cox2
Actin
LPS:
01037:
Cox2 protein relative to Actin
0.5
0.4
0.3
0.2
0.1
0.0
Ctl+DMSO
Ctl+01037
UCP2 kd+DMSO
UCP2 kd+01037
D
iNOS
Actin
LPS:
+IFNγ
01037:
iNOS protein relative to Actin
0.20
0.15
0.10
0.05
0.00
E
UCP2-2 kd
iNOS protein normalized to Actin
UCP2-2 kd+DMSO
UCP2-2 kd+01037

**Figure 5**

**UCP2 up-regulation mediates the decreased inflammation in FABP4/aP2 deficient macrophages.** (A) Cyclooxygenae 2 (Cox2) abundance measured by western blot in WT and AKO macrophages co-treated with or without LPS (100 ng/ml) ± genipin (40 μM) for 18 hours. (B) Secreted TNFα in cell culture medium determined by ELISA in WT and AKO macrophages co-treated with or without LPS (100 ng/ml) ± genipin (40 μM) for 8 hours. (C) Cox2 abundance determined by western blot in UCP2 knockdown and control macrophages pretreated with vehicle or HTS01037 (30 μM) for 3 hours and then treated with LPS (100 ng/ml) for 12 hours. (D-E) iNOS abundance determined by western blot in UCP2 knockdown and control macrophages pretreated with vehicle or HTS01037 (30μM) for 3 hours and then treated with LPS (100 ng/ml) +IFN-γ (10U) for 12 hours (UCP2 kd) and 4 hours (UCP2-2 kd) macrophages (* $p<0.05$, ** $p<0.01$)

**UCP2 decreases the hydrogen peroxide level and oxidative stress in FABP4/aP2 deficient macrophages.** A well-defined role for UCP2 is suppression of reactive oxygen species (ROS) production (13, 14). In order to determine the effect of FABP4/aP2 loss on ROS level in macrophages, the intracellular hydrogen peroxide levels in both FABP4/aP2$^{-/-}$ and wild type macrophages were determined. Figure 6A shows that FABP4/aP2$^{-/-}$ macrophages have a significantly lower level of intracellular hydrogen peroxide. Moreover, treatment of control and FABP4/aP2$^{-/-}$ macrophages with genipin to inhibit UCP2 attenuated the decreased ROS levels in FABP deficient cells but had little effect on control macrophages (Fig. 6B). Interestingly, HTS01037 treatment of Raw264.7 macrophages, which mimics the knockdown of FABP4/aP2, also led to decreased intracellular hydrogen peroxide (Fig. 6C). Moreover, HTS01037 treatment of UCP2 knockdown cells was not able to reduce the intracellular hydrogen peroxide level (Fig. 6D) suggesting the effect of HTS01037 treatment on hydrogen peroxide level is likely to be mediated by UCP2 expression as well. Taken together, the results indicate that genetic loss or chemical inhibition of FABP4/aP2 leads to the reduced level of intracellular hydrogen peroxide in an UCP2-dependent manner.

Oxidative stress is a key contributor to mitochondrial dysfunction and apoptosis (33, 34). Consistent with decreased hydrogen peroxide that is indicative of reduced oxidative stress, most of the antioxidants, if not all, are decreased in FABP4/aP2 deficient macrophages (Fig. 6E). Surprisingly, silencing of UCP2

also leads to a decrease in the mRNA expression for several antioxidant enzymes (Fig. 6E and F). One of the effects of oxidative stress is protein carbonylation, the covalent modification of proteins with reactive lipid aldehydes that is linked to mitochondrial dysfunction (35). Utilizing an antibody directed to carbonylated proteins, and consistent with reduced ROS levels, FABP4/aP2$^{-/-}$ macrophages exhibit reduced protein carbonylation (Fig. 6G).

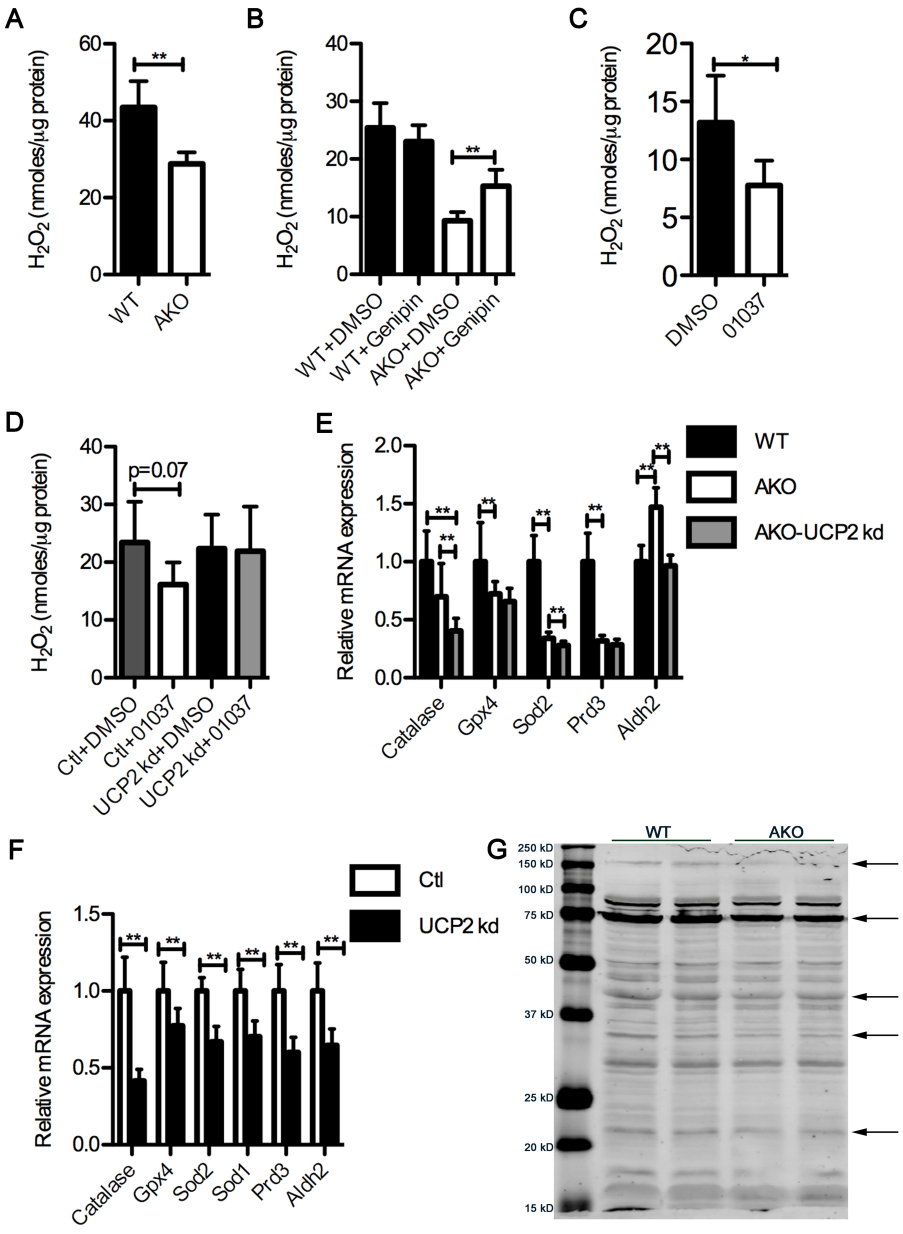
A
B
C
D
E
F
G
$H_2O_2$ (nmoles/μg protein)
WT
AKO
WT+DMSO
WT+Genipin
AKO+DMSO
AKO+Genipin
DMSO
01037
p=0.07
Ctl+DMSO
Ctl+01037
UCP2 kd+DMSO
UCP2 kd+01037
Relative mRNA expression
AKO-UCP2 kd
Catalase
Gpx4
Sod2
Prd3
Aldh2
Ctl
UCP2 kd
Sod1
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD
15 kD

**Figure 6**. **UCP2 up-regulation decreases intracellular hydrogen peroxide in FABP4/aP2 deficient macrophages.** Intracellular hydrogen peroxide level measured by Amplex Red Assay in (A) WT and AKO macrophages, (B) WT and AKO macrophages treated with vehicle or genipin (50 μM) for 6 hours, (C) Raw264.7 macrophages treated with HTS01037 (30 μM) for 24 hr and (D) UCP2 knockdown and control cells treated with HTS01037 (30 μM) for 6 hours. Expression of antioxidant gene mRNA levels in (E) WT, AKO, AKO-UCP2 kd cells and (F) UCP2 knockdown and control cells. (G) Protein carbonylation measured by western blot with anti-HNE antibody in WT and AKO mitochondrial protein. (* $p<0.05$, ** $p<0.01$)

**FABP4/aP2$^{-/-}$ macrophages have higher mitochondrial respiration capacity.** Reduced carbonylation is predictive of improved mitochondrial function and to test this hypothesis, cellular respiration was evaluated both at basal level and in response to a LPS challenge. As shown in Figure 7, FABP4/aP2$^{-/-}$ macrophages exhibited a lower level of basal respiration, and ATP turnover, but a significantly higher level of maximum respiration. Upon LPS treatment, FABP4/aP2$^{-/-}$ macrophages have a significant increase of basal respiration and ATP turnover, while wild type macrophages lose the maximum respiration capacity (Fig. 7B-D). However, no difference of coupling efficiency and proton leak between the two cell lines or treatment was observed (Fig. 7E and F).

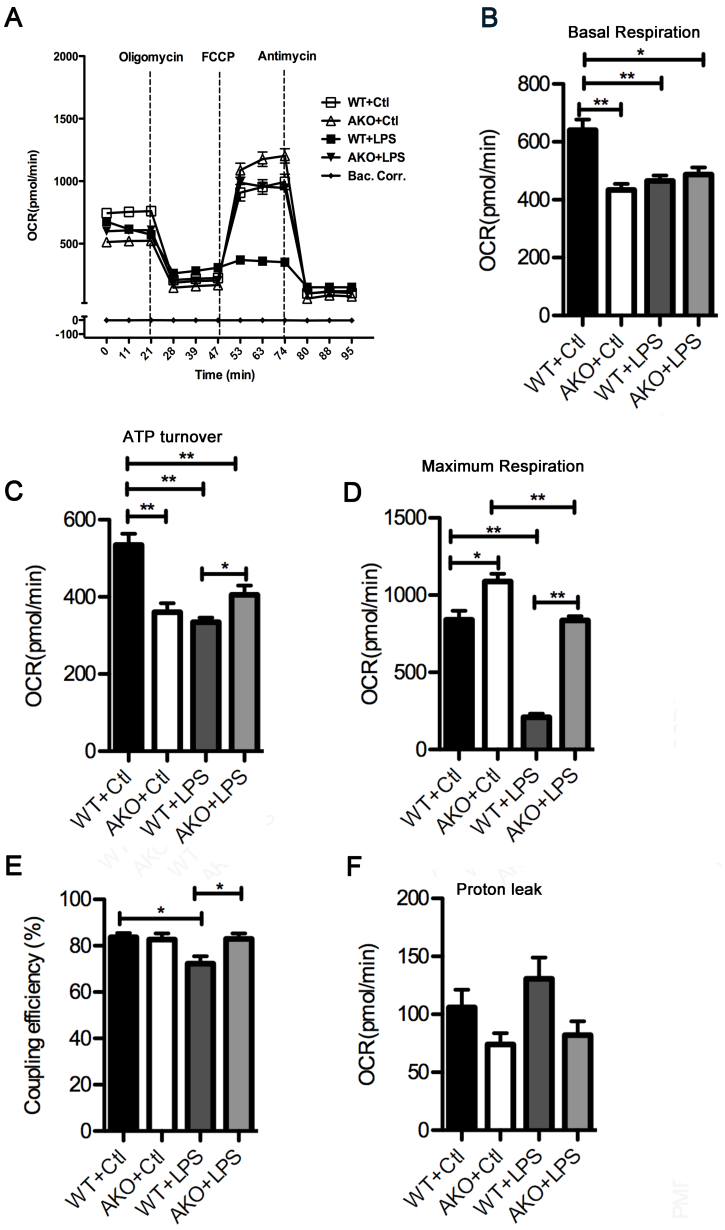


**Figure 7**. **Cellular respiration of FABP4/aP2 deficient and wild type macrophages.** (A-F) Oxygen consumption rates in WT and AKO macrophages treated with or without LPS (100 ng/ml) for 4 hours (oligomycin, 2 μM; FCCP, 0.4 μM; antimycin, 4 μM). (* $p<0.05$, ** $p<0.01$)

**UCP2 mediates decreased basal respiration and lactate production of FABP4/aP2$^{-/-}$ macrophages but not the increased fatty acid oxidation.**

Consistent with a role of UCP2 in control of basal respiration, silencing of UCP2 in FABP4/aP2$^{-/-}$ macrophages increased basal, but not maximal respiration compared to control cells (Fig. 8A). Additionally, fatty acid oxidation was increased in macrophages lacking FABP4/aP2 (Fig. 8B). However, no difference of fatty acid oxidation was observed in UCP2 knock down FABP/aP2$^{-/-}$ or Raw264.7 macrophages (Fig. 8B and C) suggesting an UCP2-independent process. FABP4/aP2$^{-/-}$ macrophage cell culture medium had reduced lactate compared to wild type macrophages (Fig. 8D). This is consistent with the lower basal respiration of FABP4/aP2$^{-/-}$ macrophages (Fig. 7B). In support of the role of UCP2 mediating the decreased lactate production in FABP4/aP2$^{-/-}$ macrophages, the lactate level was increased about 70% in the cell culture medium of UCP2 knock down FABP4/aP2$^{-/-}$ cells and UCP2 knockdown Raw264.7 cells compared with control macrophages (Fig. 8D and E).

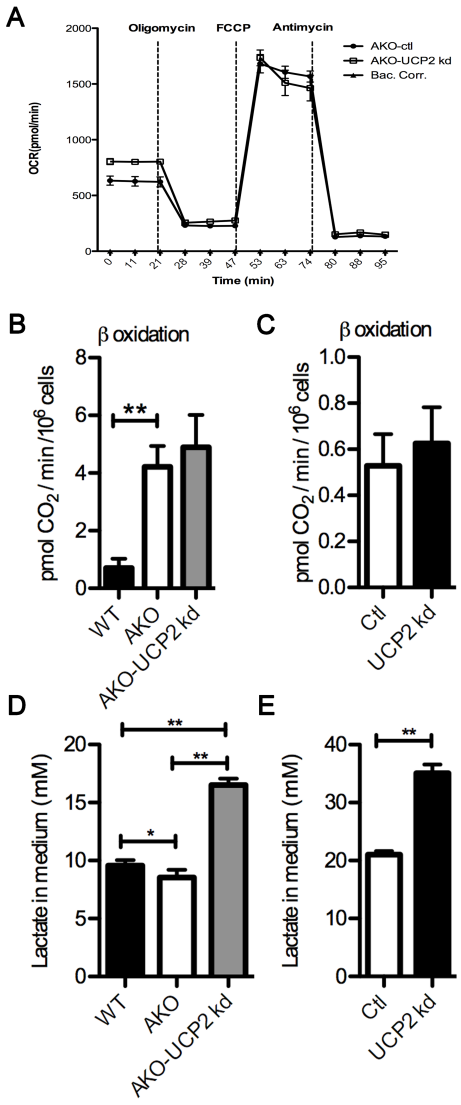


**Figure 8. Metabolic impact of UCP2 silencing on macrophages.**

(A) Oxygen consumption rate in FABP4/aP2 deficient and AKO-UCP2 kd macrophages. (B) β-oxidation measured in WT, AKO, AKO-UCP2 kd cells. (C) β-oxidation measured in UCP2 kd cells and control cells. (D) Cell culture media lactate level measured in WT, AKO, AKO-UCP2 kd cells. (E) Cell culture media lactate level measured in UCP2 knockdown and control cells. (* $p<0.05$, ** $p<0.01$)

## DISCUSSION

Chronic activation of ER stress and inflammation in macrophages contributes to the pathogenesis of various metabolic disarrangements such as atherosclerosis and type 2 diabetes (4, 29). The stressors that can lead to macrophage ER stress and inflammation include oxidative stress, high level of intracellular cholesterol and saturated fatty acids (29). Prolonged elevation of macrophage ER stress and inflammation has been proposed to contribute to macrophage apoptosis and lead to plaque necrosis and rupture (2, 36). Therefore, understanding of biological processes involved in counteracting macrophage ER stress and inflammation is crucial for development of specific strategies to improve metabolic poise. Interestingly, FABP4/aP2 has been shown to play an important role in mediating both ER stress and inflammation in macrophages (6, 37). Genetic ablation or chemical inhibition of FABP4/aP2 alleviates macrophage inflammation and ER stress (6, 8, 10). The increase of monounsaturated fatty acids, PPARγ, and LxRα activity have all been suggested as mediating the anti-inflammatory and anti-ER stress effects of FABP4/aP2 deficiency (6, 10). However, the molecular relationship of FABP deficiency to inflammation and ER stress outcome is still unclear. Increased expression of UCP2 in FABP4/aP2$^{-/-}$ macrophages provides a mechanistic basis for the anti-inflammatory, anti-ER stress outcomes. Studies in a number of systems have implicated UCP2 as a major control element in macrophage ER stress, inflammation and diet induced atherosclerosis (38-40). Interestingly, a UCP2 promoter region -866G>A polymorphism which decreases UCP2 expression has been associated with

increased risk of obesity, decreased insulin level and type 2 diabetes (14). Moreover, type 2 diabetes patients bearing the G allele have a higher inflammatory status (41). In a second study, a UCP2 -86G>A polymorphism is associated with multiple chronic inflammatory diseases, including Crohn's disease, ulcerative colitis, and psoriasis (42).

It has been proposed that unsaturated fatty acids (or their metabolites) are potential ligands for LxRα and PPARγ (27). Fatty acids and the expression of LxRα and PPARγ are increased in FABP4/aP2 deficient macrophages and have all been previously shown to up-regulate UCP2 expression (14). Interestingly, a previous study by Erbay et al. showed that the unsaturated fatty acid pool is increased in FABP4/aP2$^{-/-}$ macrophages (6). Results herein show that monounsaturated fatty acids are increased in FABP4/aP2$^{-/-}$ macrophages, and unsaturated, but not saturated, fatty acids induce UCP2 expression in macrophages, consistent with a report on fatty acid induction of UCP2 in liver cells (43). Moreover, given the increase in the intracellular free fatty acid pool in FABP4/aP2$^{-/-}$ macrophages, it is very likely they directly or indirectly activate PPARγ, thereby inducing UCP2 expression. However, this does not exclude the possibility that fatty acids may also directly regulate UCP2 activity.

Paradoxically, despite the existence of the other FABP in macrophages (FABP5/mal1), the deficiency of FABP4 leads to an anti-inflammatory and anti-atherosclerotic phenotype (7). One explanation is that FABP4 has a higher

affinity for unsaturated fatty acids compared to FABP5 (44). Therefore, the loss of FABP4 will affect the pool of available free unsaturated fatty acids, especially monounsaturated fatty acids. An additional possibility is the pools of fatty acids bound to FABP4 are distinct from those bound to FABP5, such that loss of FABP4 could exhibit a unique phenotype. Experiments to test these hypotheses are currently underway.

The reduced ER stress and inflammation demonstrated in both FABP4/aP2 deficient and FABP4/aP2 inhibited macrophages was markedly abolished or compromised by knockdown or inhibition of UCP2. This observation places UCP2 as a modulator between the FABP-FFA equilibrium and ER stress and inflammation, which is a role for UCP2 that has previously not been appreciated. It is noteworthy that the findings do not rule out contributions from other parallel pathways that reduce ER stress and inflammation. However, the results imply that the UCP2 mediated pathway is likely the major determinant of the FABP4/aP2 deficient macrophage phenotype since UCP2 knockdown or inhibition greatly compromised the protection from loss of FABP4/aP2 function.

The prominent and well-defined role of UCP2 is to suppress ROS (13). The data herein show that FABP4/aP2$^{-/-}$ macrophages have a lower level of intracellular hydrogen peroxide that is dependent on UCP2 expression as evidenced by restoration via genipin inhibition of UCP2. Hydrogen peroxide has been shown to directly oxidize the side chains of several amino acids, particularly cysteine,

thereby affecting the cellular redoxome. Alternatively, if not detoxified by antioxidants such as catalase, peroxiredoxin, or glutathione peroxidase, hydrogen peroxide could react with iron to form hydroxyl radicals and oxidize lipids. 4-hydroxynonenal (4-HNE) and 4-oxononenal (4-ONE) are the two most well studied lipid peroxidation products and have been shown to covalently modify proteins, a process termed as protein carbonylation. This frequently leads to loss or alteration of protein activity and mitochondrial dysfunction (35, 45). Interestingly, a decreased level of mitochondrial protein carbonylation was observed in FABP4/aP2$^{-/-}$ macrophages consistent with improved mitochondrial function (35). The results in Figure 7 demonstrating that FABP4/aP2$^{-/-}$ macrophages were protected from LPS-induced loss of mitochondrial respiration capacity support this concept. Interestingly, it has been shown that LPS induced mitochondrial dysfunction mainly relies on the increased ROS production and suppression of ROS not only protects cells from LPS induced mitochondrial dysfunction but also greatly attenuates LPS induced inflammatory responses (46, 47). Further work is still required to determine if the decreased hydrogen peroxide level in FABP4/aP2$^{-/-}$ macrophages is responsible for the protection from LPS induced mitochondrial dysfunction.

Both hydrogen peroxide and oxidized lipids have also been shown to activate ER stress and inflammatory pathways (48, 49). It is tempting to speculate that the UCP2 suppression of ROS in FABP4/aP2$^{-/-}$ macrophages broadly impacts not only mitochondrial function but also endoplasmic reticulum function and

inflammatory pathway activity (Fig. 9). Interestingly, knockdown of UCP2 in macrophages increases lactate production. The increased lactate production indicates increased energy production through glycolysis, a common indicator of electron transport chain dysfunction (50). Additionally, this may also explain UCP2 dependency of the lower basal respiration of FABP4/aP2 deficient macrophages. As a suppressor of ROS production, higher UCP2 expression in FABP4/aP2$^{-/-}$ macrophages leads to lower cellular oxidative stress. Since oxidative stress drives the up-regulation of antioxidants, it is reasonable that most genes linked to antioxidant biology are expressed at lower levels in FABP4/aP2$^{-/-}$ cells compared to wild type macrophages (Fig. 6E). The reduced UCP2 expression in macrophages, which leads to increased oxidative stress, would be predicted to lead to increased antioxidant protein expression. Paradoxically, in the FABP4/aP2$^{-/-}$, UCP2 kd macrophages, expression of antioxidant enzymes are down-regulated. This suggests UCP2 is involved in a more complex regulation of antioxidant expression. Nevertheless, the loss of antioxidant capacity of UCP2 knock down is consistent with a previous report showing that UCP2 deficient mice have reduced antioxidant capacity (38).

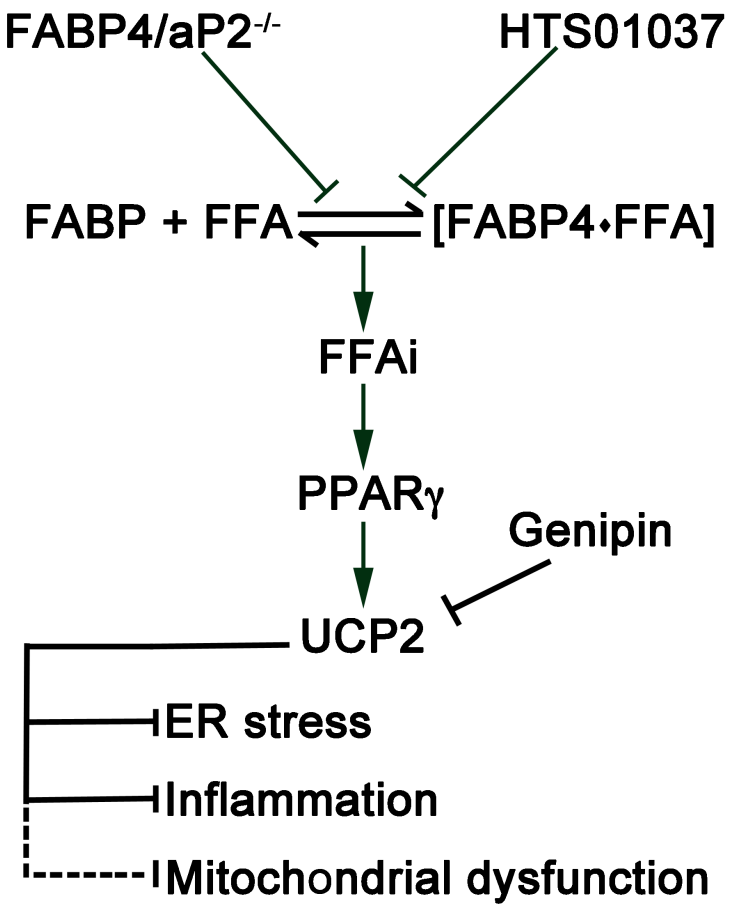


**Figure 9**. **Schematic model of the role of UCP2 up-regulation in macrophages.** Loss or inhibition of FABP4/aP2 increases the intracellular free fatty acid levels and induces expression of UCP2 via a PPAR-γ mediated pathway. Increased expression of UCP2 reduces oxidative stress in the mitochondrion, alleviates ER stress, inflammation and alters mitochondrial function in macrophages. FFAi refers to intracellular free fatty acids.

Overall, the studies herein provide a mechanistic basis for the metabolic improvement in FABP4/aP2 deficient cells generated by either genetic or pharmacologic means. Loss of FABP-FFA equilibrium is likely to increase the bioavailability of fatty acids, particularly unsaturated lipids that have the potential to increase the expression of UCP2. Whether this mechanism exists in non-macrophage cells and would pertain to the metabolic changes observed in FABP1, FABP2 or FABP3 null mice remains unknown (51). However, it may be that FABP-dependent modulation of intracellular FFA levels and therefore UCP2 expression may be a common property in many cell types.

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# CHAPTER THREE

## Loss of Fatty Acid Binding Protein 4/aP2 Reduces Macrophage Inflammation Through Activation of SIRT3

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Kaylee Steen helped prepare animal tissue samples in figure 1 and helped in the editing process of this manuscript

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**SUMMARY**

Activation of pro-inflammatory macrophages plays an important role in the pathogenesis of insulin resistance, type 2 diabetes and atherosclerosis. Prior work using high-fat fed mice has shown that ablation of the adipocyte fatty acid binding protein (FABP4/aP2) in macrophages leads to an anti-inflammatory state both in situ and in vivo and the mechanism is linked, in part, to increased intracellular monounsaturated fatty acids and the up regulation of UCP2. Herein, we show that loss of FABP4/aP2 in macrophages additionally induces SIRT3 expression and that monounsaturated fatty acids (C16:1, C18:1) lead to increased SIRT3 protein expression. Increased expression of SirT3 in FABP4/aP2 null macrophages occurs at the protein level with no change in SirT3 mRNA. When compared to controls, silencing of SIRT3 in Raw246.7 macrophages leads to increased expression of inflammatory cytokines, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (Cox2). In contrast, loss of SIRT3 in FABP4/aP2 deficient macrophages attenuates the suppressed inflammatory signaling, reduced reactive oxygen (ROS) production, LPS induced mitochondrial dysfunction and increased fatty acid oxidation. These results suggest that the anti-inflammatory phenotype of FABP4/aP2 null mice is mediated by increased intracellular monounsaturated fatty acids leading to the increased expression of both UCP2 and SirT3.

## INTRODUCTION

The prevalence of syndromes associated with obesity, including insulin resistance, hypertension and dyslipidemia have increased over the last decade (1,2). High saturated fat or “western” diets and lack of exercise contribute to the epidemic of the metabolic syndrome (1,3,4). At the molecular level, multiple pathways are involved in the pathogenesis of metabolic diseases including lipotoxicity and chronic inflammation in multiple tissues such as liver, muscle, and adipose (5-8). Importantly, the infiltration and activation of immune cells such as T cells and macrophages play an essential role in the development of insulin resistance in adipose tissue (9,10). Of these activated immune cells, macrophages play an integral role in the production of inflammatory cytokines as well as in the development of oxidative stress in adipose tissue (8).

Macrophage lipid metabolism is critical in mediating adipose tissue inflammation and oxidative stress (11,12). Work from our laboratory and many others have shown that the adipocyte fatty acid binding protein (FABP4, also know as aP2) plays an important role in the activation of macrophage inflammation (13-16). Ablation of FABP4/aP2 (AKO) in macrophages alone is sufficient to protect mice from diet induced atherosclerosis and dyslipidemia (14,16). FABP4/aP2 is a small 15 kDa lipid chaperone involved in intracellular fatty acid trafficking but recently has been shown to be secreted into the extracellular environment (17,18). Deficiency of FABP4/aP2 leads to suppressed inflammation, decreased ER stress and decreased NF-κB activation in macrophages (13,19,20).

Consistent with its role in ameliorating metabolic disorder in mice, a genetic variant at the promoter region of human FABP4/aP2, which leads to decreased expression, has been associated with reduced risk of coronary disease and type 2 diabetes (21).

Previous work has shown that the monounsaturated fatty acids palmitoleate and oleate are specifically increased in FABP4/aP2 deficient macrophages and are linked to the selective up regulation of uncoupling protein 2 (UCP2) (22). Increased expression of UCP2 in macrophages attenuates oxidative stress and reduces inflammatory signaling. However, increased UCP2 expression alone is not sufficient to explain the increased fatty acid oxidation and resistance from lipopolysaccharide (LPS) induced mitochondrial dysfunction in FABP4/aP2 deficient cells suggesting additional, unappreciated regulatory mechanisms. Herein we report that SIRT3, a member of the sirtuin family, is specifically up regulated in macrophages deficient in FABP4/aP2 and that monounsaturated fatty acids can induce SIRT3 up regulation at the protein level. The resulting increase of SIRT3 expression in macrophages is linked to suppressed inflammatory signaling as well as decreased reactive oxygen production and increased fatty acid oxidation in FABP4/aP2 deficient macrophages. Furthermore, SIRT3 up regulation protects FABP4/aP2 deficient macrophages from LPS induced mitochondrial dysfunction.

## MATERIALS AND METHODS

**Cell culture.** Raw264.7 macrophages and Sirt3 knockdown Raw264.7 macrophages were maintained in DMEM (Invitrogen) with 10% fetal bovine serum (FBS). FABP4/aP2 knockout (AKO), wild type and Sirt3 knock down FABP4/aP2 deficient macrophages were maintained in RPMI 1640 (Invitrogen) with 5% FBS.

**Quantitative RT-PCR.** Total RNA was isolated using TRIzol reagent (Invitrogen). cDNA synthesis was performed by using iScript according to the manufacturer's protocol (Bio-Rad). qRT-PCR amplification utilized a Bio-Rad CFX 96 real-time system with a SYBR green Supermix (Bio-Rad). Transcription factor II E (TFIIE) was used as an internal control to normalize expression. Primer sequences are provided in Table 1.

**Silencing of Sirt3 in macrophages.** Raw264.7 macrophages were transduced with a short hairpin RNA (shRNA) lentivirus targeting either Green Fluorescent Protein (GFP) or SirT3 as described previously (23). Green fluorescent protein and Sirt3 (GenBank accession number NP_001120823) targeting sequences were obtained from Open Biosystems: Sirt3 (kd-1), 5′-CCGGGCCATCTTTGAACTTGGCTTTCTCGAGAAAGCCAAGTTCAAAGATGGCTTTTTG-3’; GFP, 5′-AACGTACGCGGAATACTTCGA-3′. Because SIRT3 is highly expressed in FABP4/aP2$^{-/-}$ macrophages (AKO) compared to wild type cells, two rounds of lentiviral infection was required to obtain significant silencing.

To that end, FABP4/aP2$^{-/-}$ macrophages were first transduced with kd-1 followed by an additional shRNA lentivirus (kd-2), 5′-CCGGCCTACTCCATATGGCTGACTTCTCGAGAAGTCAGCCATATGGAGTAGGTTTTTG-3′.

**Stromal vascular fraction isolation.** Isolation of the stromal vascular fraction was performed as described in Xu et al. (29). Briefly, epididymal fat pads were dissected from wild type and FABP4/aP2 knockout (AKO) mice (15 week old male C57BL/6J mice maintained on high saturated fat diet (BioServe F3282) for 12 weeks), minced and digested in Krebs-Ringers-HEPES (KRH) buffer supplemented with type I collagenase (Worthington) and bovine serum albumin (BSA) for one hour at 37° C. The mixture was filtered with 100-μm-pore-size nylon cell strainer (Falcon) to remove undigested tissue. The stromal vascular fraction (SVF) was collected by centrifugation at 500 × *g* for 10 min. After washing with KRH buffer, the SVF was either resuspended in TRIzol reagent for RNA isolation or in cell lysis buffer supplemented with protease inhibitors for protein assays. All experimental procedures using animals were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee.

**Mitochondrial isolation and β-oxidation.** Mitochondrial isolation were carried out as described in Xu et al. (29). Briefly, cells were scraped into ice-cold mitochondrial isolation buffer (20 mM Tris pH 7.4, 220 mM mannitol, 70 mM

sucrose, 1 mM EDTA, 0.1 mM EGTA) and supplemented with protease inhibitors. Cells were then lysed with 20 strokes of a Dounce homogenizer and homogenates centrifuged at 700 × *g* for 10 min to remove nuclei and unbroken cells. Mitochondria were pelleted by centrifugation at 10,000 × *g* for 15 min at 4° C. Fatty acid oxidation was carried out as described previously (29).

**Measurement of ROS.** ROS production was measured by incubating cells with cell permeable 2', 7'-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) (Invitrogen). Briefly, cells were washed with PBS, and incubated in 1 mL of KRH buffer (pH 7.4) with 10 μM final concentration of $H_2$DCFDA for 30 minutes. Then cells were washed with PBS and harvested into 300 μL KRH buffer. Of each sample, 150 μL was loaded into a 96-well plate and fluorescence was measured using a microplate reader with excitation at 488 nm and emission at 535 nm.

**Immunoblot analysis.** Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors (Calbiochem). 50 μg of protein from each sample were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with Odyssey blocking buffer (Li-Cor Biosciences), membranes were incubated with primary antibody overnight at 4° C. Membranes were washed and incubated with secondary antibody conjugated to Li-Cor IRDye for 1 h and visualized using Odyssey infrared imaging (Li-Cor Biosciences). The primary antibodies used were anti-SIRT3 (Cell Signaling), anti-FABP4, anti-cyclooxygenase-2 (anti-Cox2)

(BD Transduction Laboratories), anti-inducible nitric oxide synthase (anti-iNOS) (BD Transduction Laboratories), anti-superoxide dismutase (anti-SOD2) (Cell Signaling), anti-long-chain acyl-CoA dehydrogenase (anti-LCAD) (Abcam), anti-acetylated lysine (Cell Signaling), anti-β-actin (Sigma-Aldrich), and anti-ATP synthase-α subunit (MitoSciences).

**Immunoprecipitation.** Cells were washed twice with cold phosphate-buffered saline (PBS) and scraped into 1 ml of lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, and 1% TritonX-100) supplemented with protease inhibitors and incubated for 10 minutes at 4° C. After centrifugation at 13,500 rpm for 10 minutes, supernatants were transferred to a new tube and the protein concentration was determined. 800 µg of protein lysates were pre-cleared by incubation with 30 µL protein G beads for 4 hours. After centrifugation, supernatants were transferred to a new tube and 30 µL of a protein G bead slurry was added to each tube together with primary antibody or anti-rabbit IgG control antibody. After rotating overnight at 4° C, the pelleted beads were washed five times with 500 µL lysis buffer and resuspended in RIPA buffer. Precipitated proteins were released from the IgG beads by boiling the samples for 10 minutes and loaded onto a SDS-PAGE gel for separation.

**Cellular respiratory assay.** Macrophage respiratory assay was performed on a XF24 Analyzer (Seahorse Biosciences). Macrophages were plated on V7 microplates at a density of 200,000 cells per well a day prior to the assay. On

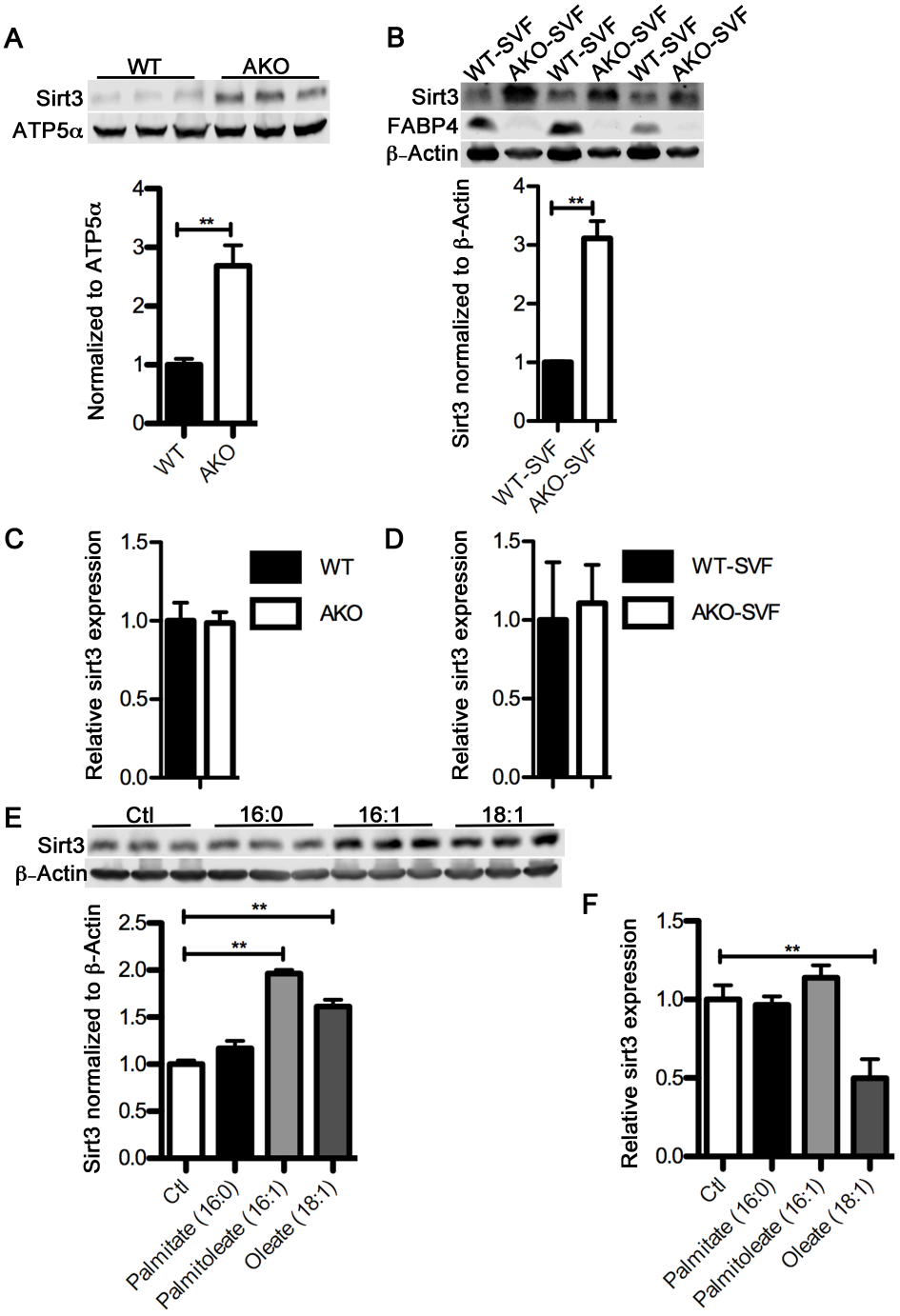
the day of the experiment, cells were treated either with vehicle or lipopolysaccharide (LPS) (100 ng/ml) for 6 hours. The cells were then washed and incubated with assay media. During the assay, cells were exposed to compounds in the following order: 2 μM oligomycin, 0.4 μM FCCP, and 4 μM antimycin A.

**Statistical analysis.** All results in the paper are expressed as the mean ± the standard error of the mean (± SEM). Statistical significance was determined using an unpaired two-tailed Student T-test.

## RESULTS

**Loss of FABP4/aP2 in macrophages increases SIRT3 expression.** Previous work by Xu et al. (22) as well as Erbay et al. and Coe et al. (20, 24) has shown that loss of FABP4/aP2 alters the intracellular level and composition of fatty acids in both macrophages and adipocytes resulting in the accumulation of monounsaturated fatty acids, particularly C16:1 and C18:1. Interestingly, consumption of monounsaturated fatty acids, which are a major component of the Mediterranean diet, are associated with improved insulin sensitivity and decreased inflammation in both humans and mice (25). In muscle, oleic acid treatment can activate the SIRT1-PGC1α (peroxisome proliferator-activated receptor γ coactivator 1-α) axis to increase fatty acid oxidation (26). More recently, Liu et al. reported that in monocytes, SIRT1 activation leads to an increase of both expression and activation of SIRT3, thus restoring

immunometabolic homeostasis (27). Therefore, we hypothesized that loss of FABP4/aP2 would increase SIRT3 expression via an increased level of monounsaturated fatty acids. To test this hypothesis, we evaluated the protein level of SIRT3 in both wild type (WT) and FABP4/aP2$^{-/-}$ (AKO) macrophages derived from wild type and FABP4/aP2$^{-/-}$ mice, respectively. SIRT3 expression in FABP4/aP2$^{-/-}$ macrophages was more than doubled compared to that in wild type (Figure 1A). To further confirm that the loss of FABP4/aP2 leads to up regulation of SIRT3 in macrophages, we isolated the stromal vascular fraction (SVF) from visceral adipose tissue of male high fat diet (HFD) fed WT and AKO mice. The level of SIRT3 was increased about three fold in AKO-SVF compared to that of wild type (Figure 1B). Interestingly, the level of Sirt3 mRNA did not change between wild type and FABP4/aP2$^{-/-}$ cell lines or SVF fractions indicating the difference of SIRT3 expression came from regulation at the translational or post-translational level (Figure 1C and D). To explore the potential role of monounsaturated fatty acids on SIRT3 expression, Raw264.7 macrophages were treated with either MUFAs (palmitoleate and oleate) or palmitate. The results showed that MUFA treatment could induce SIRT3 expression in macrophages but not the saturated fatty acid palmitate (Figure 1E). Similar to the results in experimental mice, the increase in protein was not accompanied by an increase in SirT3 mRNA (Figure 1F). Taken together, these results suggest that the increased monounsaturated fatty acids, due to the genetic ablation of FABP4/aP2, may be responsible for the increased SIRT3 expression in macrophages.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Palmitoleate (16:1)
Oleate (18:1)

**Figure 1 Loss of FABP4/aP2 increases SIRT3 expression.** (A) SIRT3 protein level normalized to ATP5α in the mitochondrial fraction of wild type (WT) and FABP4/aP2$^{-/-}$ (AKO) macrophages. (B) SIRT3 protein level normalized to β-actin in the stromal vascular fraction of epididymal adipose tissue obtained from high fat diet (HFD) fed WT and FABP4/aP2 (AKO) null mice. (C) SIRT3 mRNA level in WT and AKO macrophages. (D) SIRT3 mRNA level in the stromal vascular fraction of epididymal adipose tissue obtained from HFD fed WT and AKO mice. (E) SIRT3 protein expression in Raw264.7 macrophages treated with 300 μM palmitate, palmitoleate, or oleate for 36 hours. (F) SIRT3 mRNA level in Raw264.7 macrophages treated with 300 μM palmitate, palmitoleate, or oleate for 36 hours. Fatty acids were added in complex to bovine serum albumin at a molar ratio of 4:1 (FFA/BSA). (*$p<0.05$, **$p<0.01$, n=3-6 per group)

**SIRT3 expression is anti-inflammatory in macrophages.** Diet induced obesity is accompanied by decreased SIRT3 expression in multiple tissues including liver, skeletal muscle, heart, and pancreas (28-32). Both genetic knock out animal models and clinical studies of type 2 diabetics have shown the important role of SIRT3 in maintaining insulin sensitivity in various tissues and organs (29,30,32). However, the role of SIRT3 in tissue macrophages has not been well defined, having been studied primarily in a sepsis model (27). To illustrate the relationship between SIRT3 expression and macrophage inflammation, we silenced Sirt3 in Raw264.7 macrophages by infecting cells with lentivirus encoding a SirT3 targeted shRNA. The knockdown cell line expressed about 50% of SIRT3 protein compared to the control cells (Figure 2A). Co-treatment of Sirt3 silenced macrophage cells with LPS and interferon γ (IFNγ) induced a significantly higher level of iNOS expression in Sirt3 knockdown Raw264.7 macrophages compared to control cells (Figure 2B). In addition, macrophages treated with palmitate or palmitoleate, which have been shown to prime macrophages towards a pro- or anti-inflammatory state, respectively, regulated inflammatory marker genes. Decreased expression of SIRT3 led to increased palmitate induced Cox2 expression, and compromised the ability of palmitoleate to reduce Cox2 expression (Figure 2C). Additionally, Sirt3 knockdown Raw264.7 macrophages expressed higher transcript levels of the inflammatory cytokines TNFα and MCP1 (Figure 2D). In summary, loss of SIRT3 in macrophages results in an elevated inflammatory state.

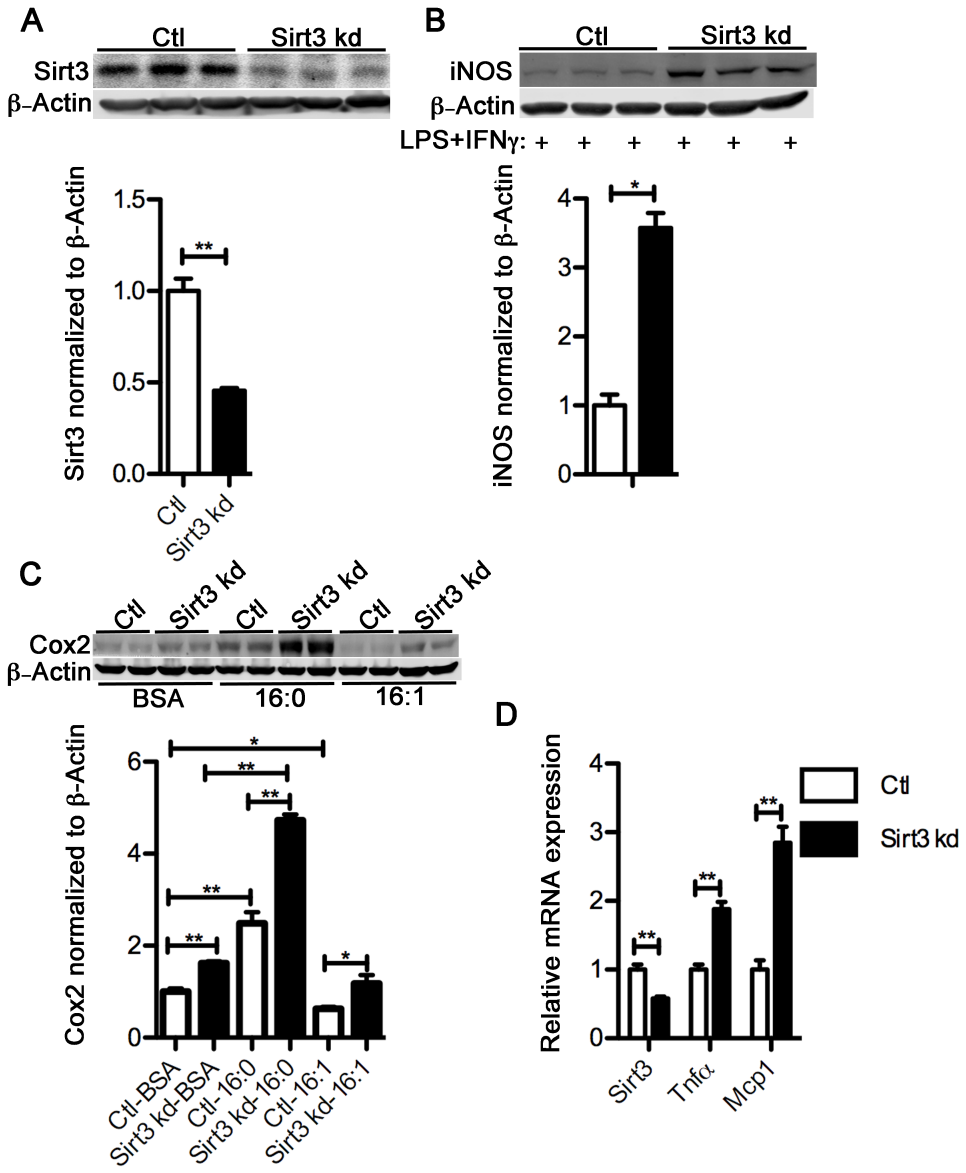
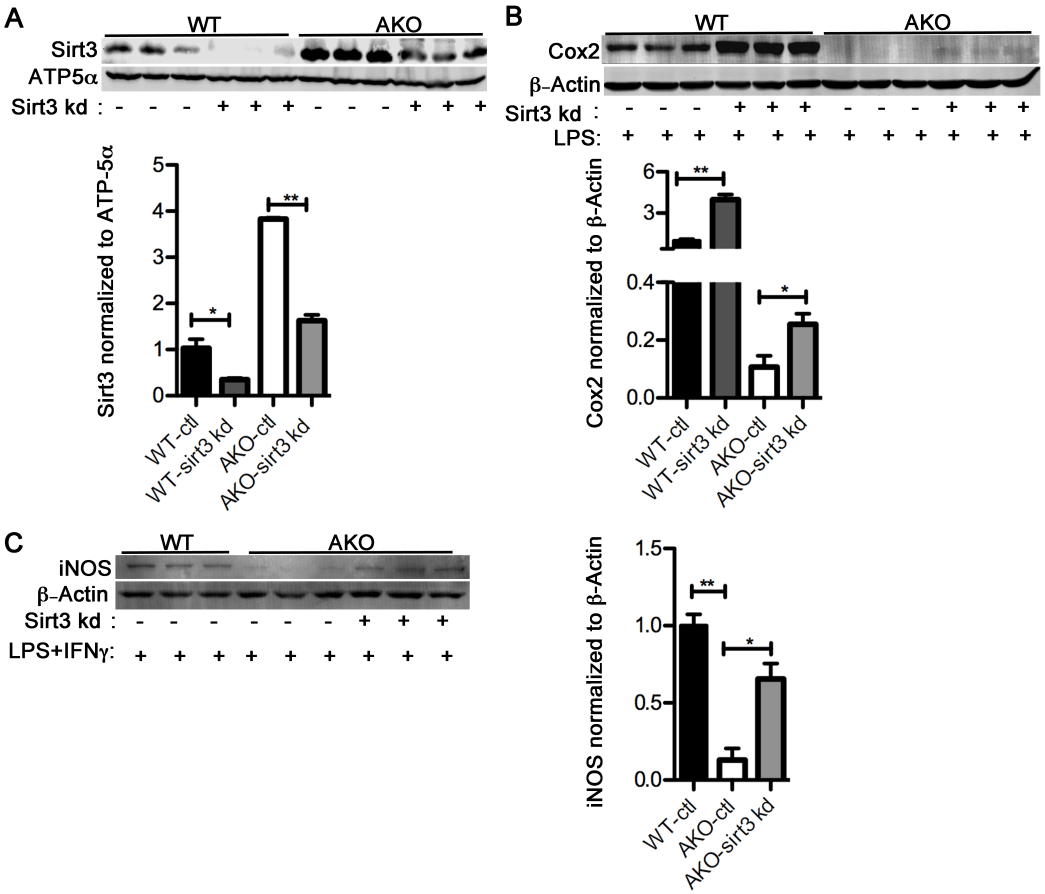



**Figure 2. Loss of SIRT3 is proinflammatory in macrophages.** (A) SIRT3 expression in SIRT3 knockdown Raw264.7 macrophages (Sirt3 kd) and control knockdown cells (Ctl). (B) iNOS abundance measured by western blot in Ctl and Sirt3 kd macrophages co-treated with LPS (100 ng/ml) and IFNγ (10U) for 14 hours. (C) Cox2 abundance in palmitate or palmitoleate (300 μM) (4:1 fatty acid to BSA) treated Ctl and Sirt3 kd macrophages. (D) mRNA levels of Sirt3, Tnfα and Mcp1 in Ctl and Sirt3 kd macrophages. (*p<0.05, **p<0.01, n=3 per group)

**SIRT3 mediates the decreased inflammatory signaling in FABP4/aP2$^{-/-}$ macrophages.** Loss of FABP4/aP2 reduces macrophage inflammatory markers such as Cox2 and iNOS (26). In order to determine if SIRT3 plays a role in the suppressed inflammatory signaling in FABP4/aP2$^{-/-}$ macrophages, we silenced SIRT3 (sirt3kd) in both wild type (WT) and FABP4/aP2$^{-/-}$ (AKO) backgrounds (Figure 3A). LPS treatment induced higher expression of Cox2 in both WT-sirt3kd and AKO-sirt3kd macrophages compared to their corresponding control cell lines. The basal expression of SIRT3 was similar between wild type and AKO-sirt3 kd macrophages, however the expression of Cox2 was still much lower in AKO-sirt3kd (Figure 3B). Therefore, SIRT3 expression in AKO is partially responsible for the reduced inflammatory signaling. Similarly, knockdown of Sirt3 in FABP4/aP2$^{-/-}$ cells dramatically increased LPS and IFNγ induced iNOS expression (Figure 3C). Additionally, genetic knockdown of Sirt3 increased the basal expression of the inflammatory cytokines, MCP1, TNFα, and MMP9 mRNA in WT and FABP4/aP2$^{-/-}$ cells compared to their respective controls (Figure 3D). In conclusion, increased SIRT3 expression in FABP4/aP2$^{-/-}$ macrophages is at least partially responsible for the reduced inflammatory signaling.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Tnfα
Mmp9
WT-ctl
WT-sirt3
AKO-ctl
AKO-sirt3

**Figure 3. SIRT3 up regulation mediates the decreased inflammation in FABP4/aP2$^{-/-}$ macrophages.** (A) SIRT3 protein expression in the mitochondrial fraction of WT-ctl, WT-sirt3 kd, AKO-ctl, and AKO-sirt3 kd macrophages. (B) Cox2 abundance determined by western blot in WT-ctl, WT-sirt3 kd, AKO-ctl, and AKO-sirt3 kd cells treated with LPS (100 ng/ml) for 16 hours. (C) iNOS abundance determined by western blot in WT-ctl, AKO-ctl and AKO-sirt3 kd cells co-treated with LPS+IFNγ for 18 hours. (D) mRNA level of SIRT3 and inflammatory cytokines in WT-ctl, WT-sirt3 kd, AKO-ctl, and AKO-sirt3 kd macrophages. (*$p<0.05$, **$p<0.01$, n=3-6 per group)

**SIRT3 mediates the decreased inflammatory signaling in FABP4/aP2$^{-/-}$ macrophages.** Loss of FABP4/aP2 reduces macrophage inflammatory markers such as Cox2 and iNOS (13). In order to determine if SIRT3 plays a role in the suppressed inflammatory signaling in FABP4/aP2$^{-/-}$ macrophages, we silenced SIRT3 (sirt3kd) in both wild type (WT) and FABP4/aP2$^{-/-}$ (AKO) backgrounds (Figure 3A). LPS treatment induced higher expression of Cox2 in both WT-sirt3kd and AKO-sirt3kd macrophages compared to their corresponding control cell lines. The basal expression of SIRT3 was similar between wild type and AKO-sirt3 kd macrophages, however the expression of Cox2 was still much lower in AKO-sirt3kd (Figure 3B). Therefore, SIRT3 expression in AKO is partially responsible for the reduced inflammatory signaling. Similarly, knockdown of Sirt3 in FABP4/aP2$^{-/-}$ cells dramatically increased LPS and IFNγ induced iNOS expression (Figure 3C). Additionally, genetic knockdown of Sirt3 increased the basal expression of the inflammatory cytokines, MCP1, TNFα, and MMP9 mRNA in WT and FABP4/aP2$^{-/-}$ cells compared to their respective controls (Figure 3D). In conclusion, increased SIRT3 expression in FABP4/aP2$^{-/-}$ macrophages is at least partially responsible for the reduced inflammatory signaling.

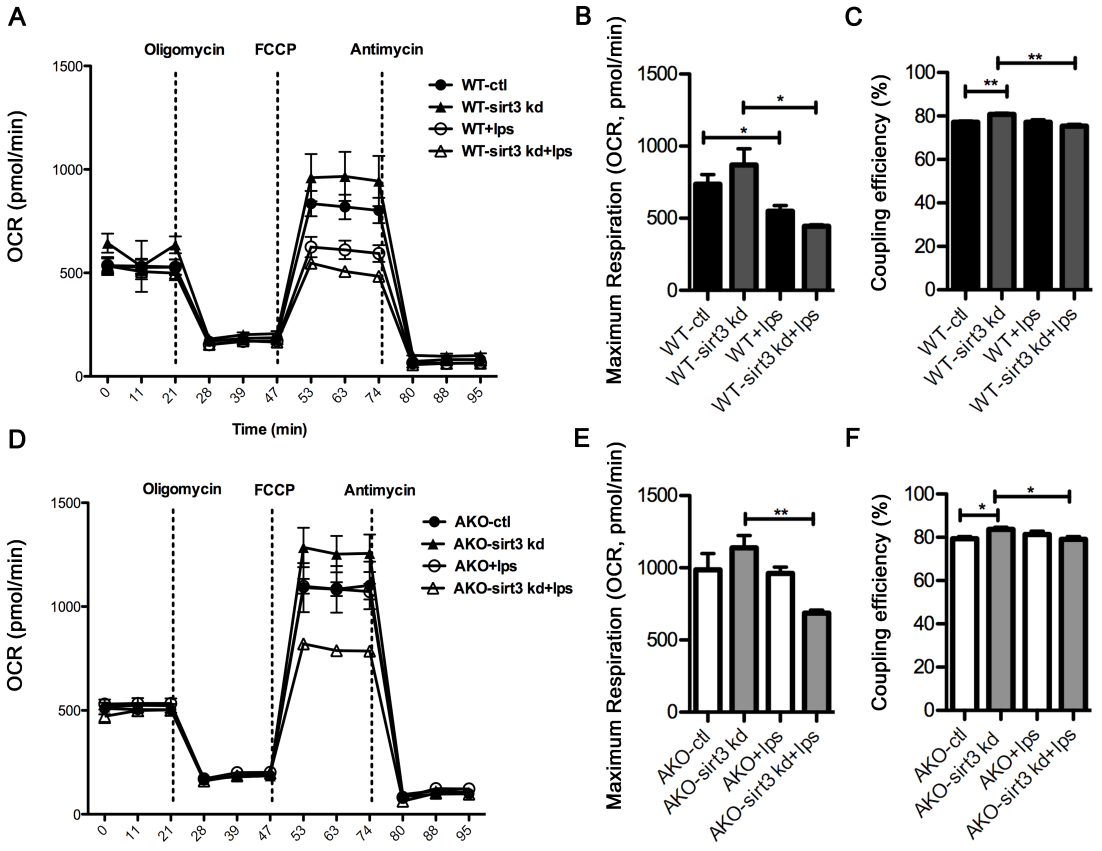


**Figure 4. SIRT3 expression protects macrophages from LPS induced mitochondrial dysfunction.** (A) Oxygen consumption rate in WT-ctl and WT-sirt3 kd macrophages treated with or without LPS (100 ng/ml) for 6 hours (oligomycin, 2 μM; FCCP, 0.4 μM; antimycin, 4 μM ). (B) Maximum respiration in WT-ctl and WT-sirt3 kd macrophages treated with or without LPS (100 ng/ml) for 6 hours. (C) Coupling efficiency in WT-ctl and WT-sirt3 kd macrophages treated with or without LPS (100 ng/ml) for 6 hours. (D) Oxygen consumption rate in AKO-ctl and AKO-sirt3 kd macrophages treated with or without LPS (100 ng/ml) for 6 hours (oligomycin, 2 μM; FCCP, 0.4 μM; antimycin, 4 μM ). (E) Maximum respiration in AKO-ctl and AKO-sirt3 kd macrophages treated with or without LPS (100 ng/ml) for 6 hours. (F) Coupling efficiency in AKO-ctl and AKO-sirt3 kd macrophages treated with or without LPS (100 ng/ml) for 6 hours. (*$p<0.05$, **$p<0.01$, n=5 per group)

**SIRT3 expression mediates the protective effect of FABP4/aP2 deficiency on LPS induced mitochondrial dysfunction.** Genetic ablation of FABP4/aP2 has been shown to protect macrophages from LPS dependent loss of maximum respiration capacity, independent of uncoupling protein 2 expression (22). In order to determine if the increased expression of SIRT3 in FABP4/aP2$^{-/-}$ macrophages could mediate the suppression of LPS induced mitochondrial dysfunction, both WT-sirt3kd and AKO-sirt3kd, as well as their control cells, were treated with LPS for 6 hours and cellular respiration was measured. Consistent with our previous report (22), LPS treatment induced a decrease of maximum respiration in wild type cells, while FABP4/aP2$^{-/-}$ macrophages were protected from this effect (Figures 4A-B and D-E). Knockdown of Sirt3 rendered wild type macrophages more susceptible to a LPS induced decrease of maximum respiration (Figure 4B). Moreover, knockdown of Sirt3 in FABP4/aP2$^{-/-}$ macrophages led to a similar reduction of maximum respiration by LPS treatment, thus eliminating the protection seen in the FABP4/aP2$^{-/-}$ macrophages (Figure 4E). Interestingly, coupling efficiency was increased after Sirt3 knockdown in wild type and FABP4/aP2$^{-/-}$ macrophages and decreased in Sirt3 knockdown cells following LPS treatment (Figures 4C and F). No difference of basal respiration or ATP turnover was observed (data not shown). Therefore, up regulation of SIRT3 in FABP4/aP2$^{-/-}$ macrophages is responsible for changes in mitochondrial respiration in response to LPS treatment.

**SIRT3 expression is responsible for fatty acid oxidation and ROS production in macrophages independent of lysine acetylation changes.**

Among the seven members in the sirtuin family, SIRT3 plays a major role in regulating mitochondrial function by affecting various aspects of mitochondrial metabolism, including mitochondrial respiration, fatty acid oxidation, and the tricarboxylic acid cycle flux (TCA cycle) (33-35). Consistent with the report that SIRT3 regulates fatty acid oxidation (34), Sirt3 knockdown in FABP4/aP2$^{-/-}$ macrophages decreased fatty acid oxidation (Figure 5A). Another prominent role of SIRT3 is controlling reactive oxygen species (ROS) production (33). Consistently, knockdown of Sirt3 increased ROS production in Raw264.7 and wild type macrophages (Figure 5B and C). Since our previous work has shown that FABP4/aP2$^{-/-}$ macrophages have a significantly lower level of oxidative stress (22), we speculated that the increased SIRT3 expression may be responsible for the decreased ROS level. Consistent with our previous report, the ROS level was significantly lower in FABP4/aP2$^{-/-}$ macrophages compared to that in the wild type (Figure 5C). Upon LPS stimulation, there was a significant increase of ROS in wild type, but not FABP4/aP2$^{-/-}$ macrophages. Importantly, upon LPS stimulation, AKO-sirt3kd cells had a significant increase in ROS production compared to AKO macrophages, however the ROS levels were still considerably lower than that in wild type macrophages in response to LPS stimulation (Figure 5C). These data indicate up regulation of SIRT3 is at least partially responsible for the decreased ROS in FABP4/aP2$^{-/-}$ macrophages in response to LPS treatment. Despite the increased SIRT3 expression in

FABP4/aP2$^{-/-}$ macrophages, we did not detect any substantive difference in the mitochondrial acetylome or the acetylation of classic SIRT3 targets, LCAD and SOD2 (Figures 5D-F).

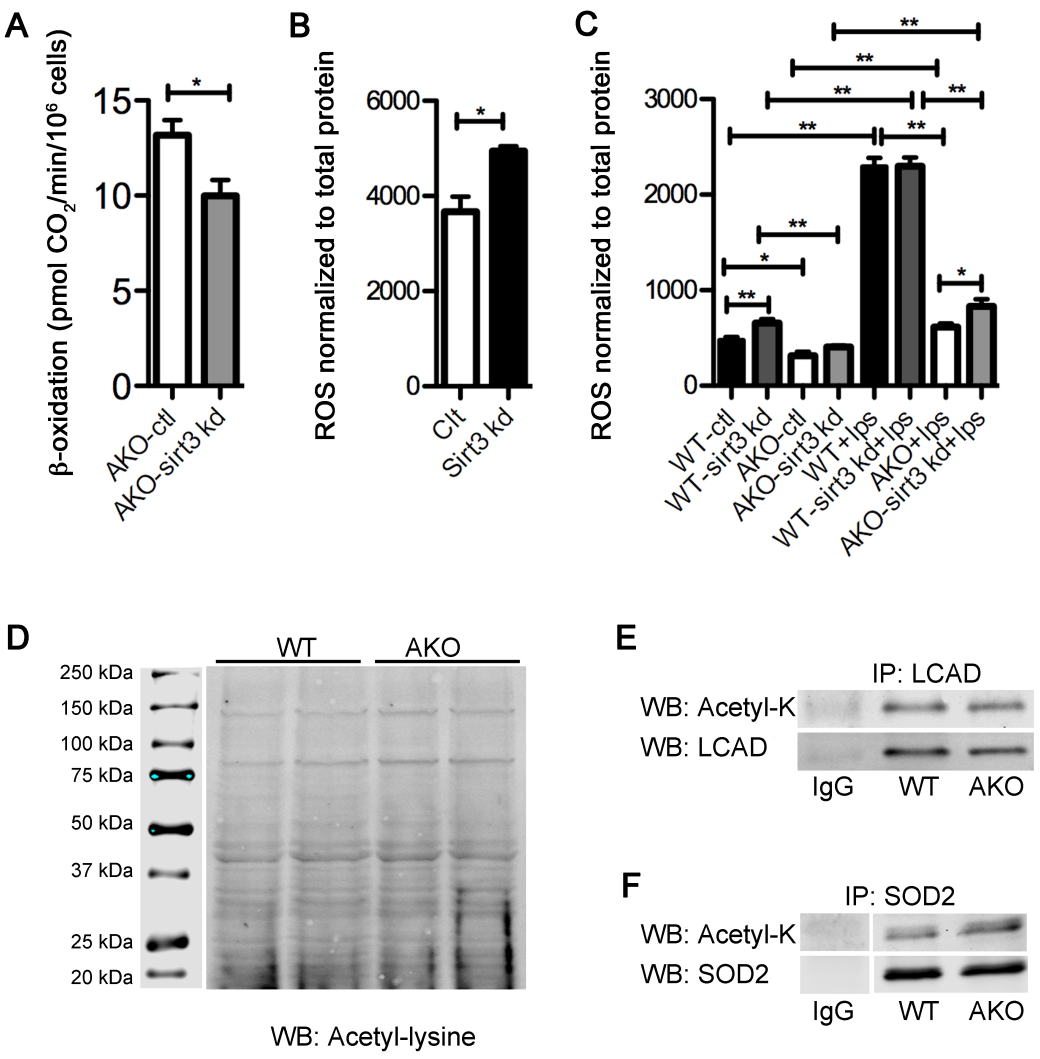


**Figure 5. Increased SIRT3 expression mediates increased β-oxidation and decreased oxidative stress in FABP4/aP2$^{-/-}$ macrophages independent of protein acetylation.** (A) β-oxidation measured in AKO-ctl and AKO-sirt3 kd macrophages. (B) ROS measured by $H_2$DCFDA in Control and Sirt3 kd Raw264.7 macrophages. (C) ROS measured by $H_2$DCFDA in WT-ctl, WT-sirt3 kd, AKO-ctl, and AKO-sirt3 kd macrophages treated with or without LPS (100 ng/ml) for 6 hours. (D) Mitochondrial protein acetylation in WT and AKO macrophages. (E) Analysis of acetylated and total LCAD following immunoprecipitation from WT and AKO macrophages. (F) Evaluation of acetylated and total SOD2 immunoprecipitated from WT and AKO macrophages. (*p<0.05, **p<0.01, n=3-6 per group)

## DISCUSSION

Insulin resistance in peripheral tissues such as liver, adipose, and muscle is a major feature in the pathogenesis of cardiovascular disease, fatty liver, and type 2 diabetes (1,5). The progression of insulin resistance is accompanied with macrophage infiltration and chronic inflammatory activation in multiple tissues (12,36-38). Mouse models which either reduce macrophage recruitment in adipose tissue or suppress macrophage inflammation have been show to protect the animal from high fat diet induced insulin resistance (39-41). One of the most well characterized mouse models that affect these inflammatory events is genetic deletion of FABP4/aP2. Despite a similar level of adiposity, FABP4/aP2$^{-/-}$ mice are protected from diet induced insulin resistance (42). Interestingly, macrophage specific deletion of FABP4/aP2 is sufficient to prevent the development of atherosclerosis in the ApoE-deficient mouse model (14). More detailed studies have shown that loss of FABP4/aP2 could suppress inflammatory signaling in macrophages, which is a major reason for the metabolic benefits of FABP4/aP2 deficiency (13,16,43,44). Recent work from our laboratory has shown that induction of UCP2 expression is a major mediator of the decreased inflammation and endoplasmic reticulum stress responses observed in FABP4/aP2$^{-/-}$ macrophages (22). However, up regulation of UCP2 does not explain the increased fatty acid oxidation and improved mitochondrial function in FABP4/aP2$^{-/-}$ macrophages. Previous reports demonstrating a MUFA-SIRT1-PGC1α-SIRT3 axis, as well as our own observation that monounsaturated fatty acids are specifically increased in FABP4/aP2$^{-/-}$ macrophages, led us to

speculate that SIRT3 expression might be altered in FABP4/aP2$^{-/-}$ macrophages (26,27). Indeed, the results herein showed a dramatic increase of SIRT3 protein, but not mRNA, following loss of macrophage FABP4/aP2. Additionally, silencing of Sirt3 in FABP4/aP2$^{-/-}$ macrophages decreased fatty acid oxidation. More importantly, our results also demonstrated for the first time that MUFAs, but not palmitate, could induce SIRT3 protein expression in macrophages. By knocking down Sirt3 in macrophages, the results here revealed an important role of SIRT3 in the suppression of macrophage inflammation. Induction of SIRT3 is required for the anti-inflammatory role of palmitoleate, as knockdown of Sirt3 compromised the reduction of Cox2 expression caused by palmitoleate treatment.

In parallel with the identification of UCP2 as a major mediator of the decreased inflammation in FABP4/aP2$^{-/-}$ macrophages, the loss of SIRT3 also restored the inflammation in FABP4/aP2 deficient cells similar to wild type macrophages. Interestingly, SIRT3 expression has been shown to be correlated with UCP2 levels in hepatocytes cultured in high glucose and in this system, inhibition of SIRT3 also leads to decreased UCP2 expression (45). Therefore, it is likely that SIRT3 acts as an upstream regulator of UCP2, and exerts some of its action by suppressing inflammation in macrophages through UCP2.

SIRT3 localizes primarily to mitochondria and regulates the activity of a number of enzymes involved in some major metabolic pathways, including tricarboxylic

acid cycle, the urea cycle, and fatty acid metabolism (33-35). Given the prominent role of SIRT3 in mitochondrial function, it was not surprising to find that the up regulation of SIRT3 is an important contributor to the improved mitochondrial function observed in FABP4/aP2$^{-/-}$ macrophages (22). While FABP4/aP2$^{-/-}$ macrophages are protected from LPS induced loss of maximum respiration capacity, knockdown of SIRT3 potentiated LPS induced mitochondrial dysfunction. Consistent with SIRT3's role as a suppressor of oxidative stress, loss of SIRT3 increased ROS production both basally and in response to LPS in macrophages.

Paradoxically, despite the dramatic up regulation of SIRT3, we were not able to detect any difference of global mitochondrial protein lysine acetylation or in the acetylation status of classic targets of SIRT3 such as LCAD and SOD2. One potential explanation is that SIRT3 exerts its role in inflammation, ROS control, and mitochondrial function through a mechanism other than protein deacetylase activity in macrophages (46). It is also possible that the acetylation status of the SIRT3 targets analyzed here are affected by other pathways in addition to SIRT3. Generally, SIRT3 is considered as a ROS suppressor mainly by regulating SOD2 acetylation status (47). However, we did not detect any difference of SOD2 expression or acetylation between wild type and FABP4/aP2$^{-/-}$ macrophages (Figure 5). While we speculate that SIRT3 serves as an oxidative stress suppressor in macrophages by regulating UCP2 expression, further work is required to validate this point.

In summary, the results presented herein demonstrate that SIRT3 is up regulated in FABP4/aP2 deficient macrophages, potentially via increased levels of palmitoleic acid. The increased SIRT3 expression mediates decreased inflammation and intracellular ROS as well as the improved mitochondrial function in FABP4/aP2$^{-/-}$ macrophages. However, increased SIRT3 expression did not affect global acetylation status of mitochondrial protein in FABP4/aP2$^{-/-}$ macrophages.

**ACKNOWLEDGMENTS**

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# CHAPTER FOUR

## FABP4/aP2 regulates macrophage redox signaling and inflammasome activation via control of UCP2

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This chapter contains an original research article previously published.

Kaylee Steen wrote this manuscript and generated the data (with the exception of Figures 1A, 4B and 4C)

**SUMMARY**

Obesity-linked metabolic disease is mechanistically associated with the accumulation of proinflammatory macrophages in adipose tissue leading to increased reactive oxygen species (ROS) production and low-grade, chronic inflammation. Previous work has demonstrated that deletion of the adipocyte fatty acid-binding protein (FABP4/aP2) uncouples obesity from inflammation via up-regulation of the uncoupling protein 2 (UCP2). Herein we demonstrate that ablation of FABP4/aP2 regulates systemic redox capacity, reduces cellular protein sulfhydryl oxidation and in particular, oxidation of mitochondrial protein cysteine residues. Coincident with the loss of FABP4/aP2 is the up-regulation of antioxidants superoxide dismutase (SOD2), catalase, methionine sulfoxide reductase A and the 20S proteasome subunits PSMB5 and αβ. Reduced mitochondrial protein oxidation in FABP4/aP2$^{-/-}$ macrophages attenuates the mitochondrial unfolded protein response (mtUPR) as measured by expression of heat shock protein 60, Clp protease and Lon peptidase 1. Consistent with a diminished mtUPR, FABP4/aP2$^{-/-}$ macrophages exhibit reduced expression of cleaved caspase 1 and NLRP3. Secretion of IL-1β, in response to inflammasome activation, is ablated in FABP4/aP2$^{-/-}$ macrophages as well as in FABP4/aP2-inhibitor treated cells but partially rescued in FABP4/aP2 null macrophages when UCP2 is silenced. Collectively, these data offer a novel pathway whereby FABP4/aP2 regulates macrophage redox signaling and inflammasome activation via control of UCP2 expression.

## INTRODUCTION

Obesity is linked with a variety of metabolic diseases including type II diabetes, dyslipidemia and cardiovascular disease. Accumulation of visceral adipose tissue (VAT) is critical for disease development as it contains inflammatory macrophages that secrete cytokines, chemokines and other signaling molecules leading to both local and systemic effects (1, 2). Increased abundance of reactive oxygen species (ROS) is one critical consequence of adipose inflammation and hydrogen peroxide ($H_2O_2$) serves as a secondary messenger in various immunometabolic signaling processes (3, 4). $H_2O_2$ leads to reversible cysteine and methionine oxidation that alters protein activity and/or interactions (5, 6). Moreover, chronically elevated oxidative species diminish the cellular pool of chemical antioxidants (GSH and NADPH) leading to a variety of pathophysiologies (7-10). Sustained protein oxidation not only leads to dysregulated signaling cascades, but also protein unfolding and potentially aggregation (11, 12). Multiple quality control systems exist in the endoplasmic reticulum and mitochondria to prevent oxidative damage and protein aggregation (4, 13-15). Despite this, the mitochondria in particular is vulnerable to sustained oxidative stress due to the high capacity for reactive oxygen species synthesis in the electron transport chain (16-18). Mitochondrial uncoupling proteins are one such mechanism to dissipate the proton gradient across the inner membrane and reduce oxidative stress (19).

In macrophages, one major consequence of mitochondrial dysfunction associated with metabolic disease is the activation of the inflammasome and secretion of IL-1β (20). Secretion of IL-1β from macrophages typically involves multiple inputs and minimally requires activation of the NF-κB pathway resulting in expression of inflammasome complex including pro-caspase-1, NACHT, LRR and PYD domains-containing protein 3 (NLRP3) and pro-IL-1β. Secondary signaling (e.g., ROS, unfolded protein response) leads to the auto-cleavage of pro-caspase-1 followed by processing of pro-IL-1β to the mature IL-1β and its subsequent secretion (21-23).

A variety of mouse models have been developed to interrogate the molecular relationship between obesity, inflammation and metabolic disease. Of these, the FABP4/aP2 knockout mouse has been intensely studied as a paradigm for regulatory systems linking lipid metabolism to inflammation. In the FABP4/aP2 system the loss of this intracellular fatty acid binding protein from macrophages results in an anti-inflammatory phenotype in both animal and cell based models (3, 24-27). At the molecular level, loss of FABP4/aP2 attenuates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, reduces c-Jun N-terminal kinase (JNK) phosphorylation, diminishes inflammatory cytokine secretion and polarizes cells from the classically activated M1 phenotype to the alternatively activated M2 (28, 29). Recently, Xu et al., reported that ablation of FABP4/aP2 in macrophages improves mitochondrial function and attenuates NF-κB signaling via monounsaturated fatty acid induction of UCP2 and sirtuin-3 (3,

28). However, the molecular mechanisms that link the increased expression of UCP2 to diminished inflammatory cytokine synthesis are unclear. To that end, in this report we describe novel findings that demonstrate the FABP4/aP2–UCP2 axis regulates mitochondrial redox biology, the mitochondrial unfolded protein response and activation of the inflammasome.

## MATERIALS AND METHODS

**Mice.** Male C57BL/6J wild type (WT) and whole body knockout of FABP4/aP2 (abbreviated as AKO) mice were fed *ad libitum* a high saturated fat (lard) diet (BioServe F3282, Flemington, NJ) from weaning for 12 weeks. Mice were used between 15-16 weeks of age and weighed 40-50 g. Such high fat fed mice developed insulin resistance as measured by impaired insulin and glucose tolerance, hyperinsulinemia, increased hepatic glucose output and elevated lipolysis (86). All experimental procedures using animals were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee.

**Cell culture.** A variety of stable cell lines linked to FABP4/aP2 biology reported herein were immortalized from bone marrow derived macrophages of chow fed WT and AKO C57BL/6J mice as previously reported (70). These include stable macrophage cells from wild type C57BL/6J mice ($WT_{M\Phi}$) and FABP4/aP2 null mice ($AKO_{M\Phi}$), wild type or AKO macrophages expressing eGFP (WT-$eGFP_{M\Phi}$, AKO-$eGFP_{M\Phi}$), and AKO macrophages in which UCP2 has been silenced (AKO-

$UKD_{M\Phi}$). All the above-mentioned macrophage cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum. Raw 264.7 control and UCP2 knockdown cells (Raw-eGFP and Raw-UKD) cells were cultured in Dulbecco's' modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (29). Bone marrow derived macrophages (BMDMs, 2 X $10^6$ cells) were isolated from C57BL/6J mice, plated and maintained in Iscove's Modified Dulbecco's Medium (Invitrogen, Carlsbad, CA), 10% FBS and 10ng/mL macrophage colony-stimulating factor (M-CSF) for one week prior to stimulation (13).

**Quantitative RT-PCR**. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) from WT and AKO macrophage cell lines. cDNA synthesis was performed by using iScript according to the manufacturer's protocol (Bio-Rad, Hercules, CA). qRT-PCR amplification utilized a Bio-Rad CFX 96 real-time system with a SYBR green Supermix. Transcription factor II E (TFIIE) and TATA-binding protein (TBP) were used as internal controls to normalize expression. Primer sequences can be found in Table I.

**Stromal vascular fraction isolation.** Isolation of stromal vascular fraction was performed as described in Xu et al (29). Visceral fat pads were dissected from wild type and FABP4/aP2 knockout mice ($WT_{SVF}$, $AKO_{SVF}$), minced and digested in Krebs-Ringers-HEPES (KRH) buffer supplemented with type I collagenase (Worthington, Lakewood, NJ) and bovine serum albumin (BSA) for one hour at

37° C. Undigested tissue was removed by filtering the mixture through 100-µm-pore-size nylon cell strainer (Fischer Scientific, Walthman, MA). The stromal vascular fraction (SVF) was collected by centrifugation at 500 × *g* for 10 min followed by two washes with KRH buffer. The newly isolated SVF was then either resuspended in TRIzol reagent for RNA isolation, in cell lysis buffer supplemented with protease inhibitors for protein assays, or precipitated with 20% TCA for cysteine oxidation detection.

**Mitochondrial isolation.** Mitochondrial isolation was carried out as described in Xu et al (29). Tissue was placed in ice-cold mitochondrial isolation buffer (20 mM Tris pH 7.4, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.1 mM EGTA) and supplemented with protease inhibitors. The tissue was then lysed with 7 strokes of a Dounce homogenizer and homogenates centrifuged at 900 × *g* for 10 min to remove nuclei and unbroken cells. To pellet the mitochondria, the supernatant was centrifuged at 10,000 × *g* for 15 min at 4° C.

**Hydrogen peroxide measurements.** Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Carlsbad, CA) was used to measure hydrogen peroxide following the manufacturer's instructions.

**Immunoblot analysis.** Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors (Calbiochem, Darmstadt, Germany). 25-50 µg of protein from each sample were separated by SDS-PAGE

and transferred to a polyvinylidene difluoride (PVDF) membrane as measured by a bicinchoninic acid (BCA) assay. After blocking with Odyssey blocking buffer (LI-COR Biosciences, Lincoln NE), membranes were incubated with primary antibody overnight at 4° C. Membranes were washed and incubated with secondary antibody conjugated to Li-Cor IRDye for 1 hr and visualized using Odyssey infrared imaging (Li-Cor Biosciences, Lincoln NE). The primary antibodies used were anti-catalase (abcam, Cambridge, MA), IRDye® 800CW Streptavidin (cysteine oxidation, LI-COR, Lincoln, NE), anti-PSMB5 (abcam, Cambridge, MA), anti-20S αβ (abcam, Cambridge, MA), anti-NLRP3 (adipogen, San Diego, CA), anti-caspase-1 (abcam, Cambridge, MA), anti-methionine sulfoxide reductase A (abcam, Cambridge, MA), anti-β-actin (Sigma-Aldrich, St. Louis, MO), anti-super oxide dismutase 2 (cell signaling, Beverly, MA), anti-LonP1 (abcam, Cambridge, MA), anti-ClpP (abcam, Cambridge, MA), anti-HSP60 (abcam, Cambridge, MA).

**Detecting global cysteine oxidation.** Wild type and AKO SVF, mitochondria isolates or macrophage cell line lysates were directly precipitated with 20% TCA for 2 hours at 4° C. The pellets were subsequently washed with 10% TCA, 5%TCA and 75% ice-cold acetone then resuspended in labeling buffer (200 mM Tris pH 8.5, 5 mM EDTA, 0.05% SDS and 6 M Urea). The lysates were labeled with 100 mM iodoacetamide for 15 minutes at room temperature followed by addition of 100% TCA (20% final) and maintained at 4° C overnight. The cell pellets were washed again and resuspended in reducing buffer (40 mM sodium

arsenite, 150 mM Tris pH 7.4, 2.5% SDS) along with 200 uM biotin-maleimide and incubated for 1hr at 50° C. Each sample was measured for protein concentration with a BCA assay and proteins were separated by SDS-PAGE. The membrane was placed in blocking buffer overnight at 4° C and incubated for 1hr in IRDye® 800CW Streptavidin to detect the cysteine conjugated biotin residues (cysteine oxidation).

**ELISA analysis.** IL-1β was detected in the media of macrophage cell lines or bone marrow derived macrophages treated with either 30 uM HTS01037 for 4 hr, 2 uM or 5 uM cyclosporin A for 1 hr, 500 ng/mL LPS for 4 hr, 10 uM MG132 for 2 hr or 2 mM ATP for 1 hr using an ELISA kit (BD Biosciences, San Jose, CA).

**Statistical analysis.** All results are expressed as standard error of the mean (± SEM). For studies using the stable wild type and AKO cell lines, results are presented from a sample size of three or more and were repeated in at least three independent experiments. For studies using cells derived from the mouse stromal vascular fraction or bone marrow derived macrophages, studies are reported from experiments performed at least 3 times with a sample size of 4-6 per experiment. Statistical significance was determined using an unpaired two-tailed Student t test.

## RESULTS

**Loss of FABP4/aP2 in macrophages induces UCP2 and reduces oxidative stress.** Prior work from Xu et al. has shown that loss of FABP4/aP2 results in elevated intracellular abundance of monounsaturated fatty acids (C16:1 and C18:1) and up regulates the expression of UCP2. Moreover, treatment of RAW264.7 cells with exogenous palmitoleic or oleic acid, but not palmitate or stearate, increased the expression of UCP2 leading to the model that monounsaturated fatty acids increase UCP2 expression (3). As shown in Figure 1A, UCP2 protein was significantly increased in FABP4/aP2 null macrophages ($AKO_{M\Phi}$) compared to control macrophages from wild type C57BL/6J mice ($WT_{M\Phi}$). Furthermore, macrophage cells that lack FABP4/aP2 either via genetic means (AKO cells) or treated with an FABP4/aP2 inhibitor (HTS01037, (26)) exhibit significantly reduced hydrogen peroxide levels (Figure 1B). AKO macrophages were also protected from the increase in hydrogen peroxide when treated with 100 ng/mL LPS for 24 hours (Figure 1C). The reduction in hydrogen peroxide was lost when UCP2 was silenced in either AKO macrophages (AKO-$UKD_{M\Phi}$) or Raw 267.4 macrophages (Raw-UKD). In addition, treatment of HTS01037 could not lower hydrogen peroxide levels in Raw-UKD cells as was observed in control Raw 264.7 macrophages (Raw-eGFP, Figure 1D-E). These data collectively indicate UCP2 is modulating the affect of reduced hydrogen peroxide in FABP4 null macrophages. This is consistent with previous studies that UCP2 can reduce ROS levels, independently from proton uncoupling (19, 32).

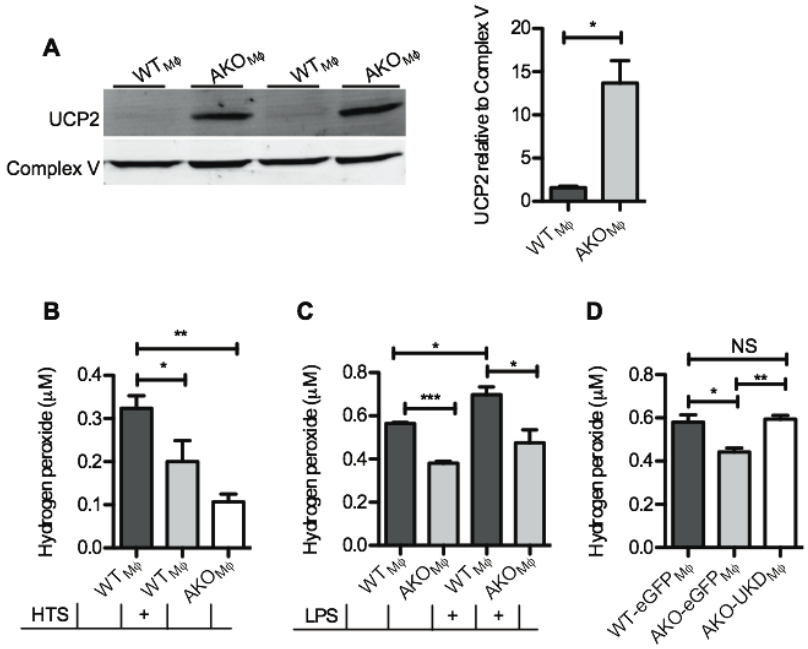
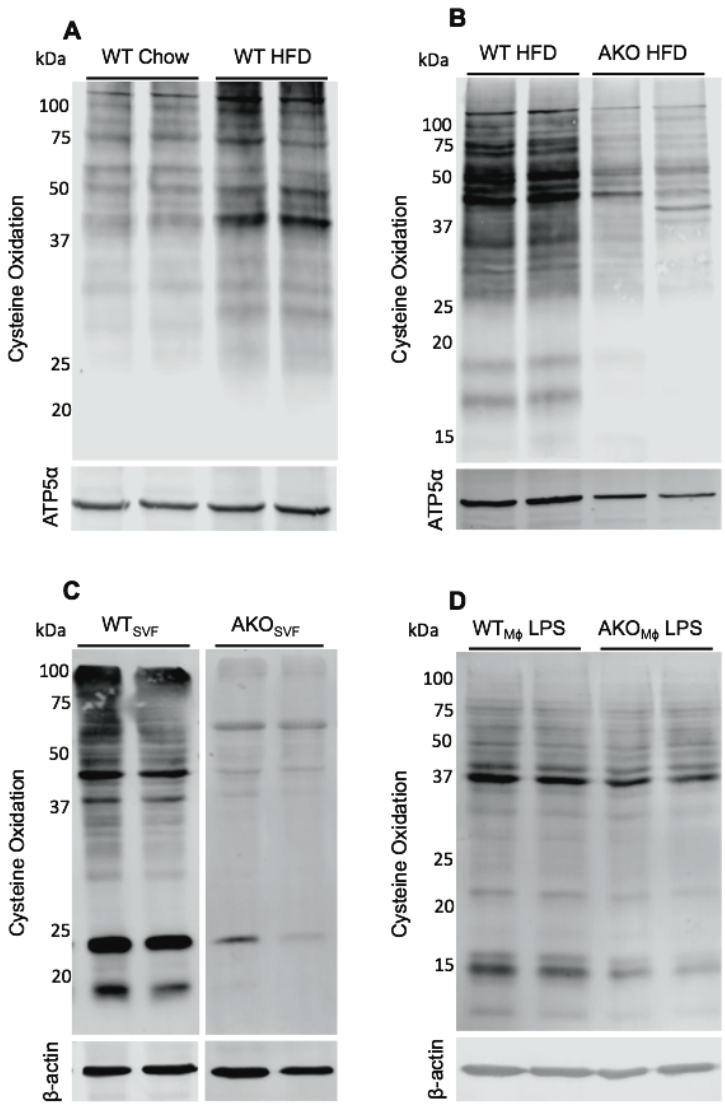



**Figure 1: Loss of FABP4/aP2 decreases hydrogen peroxide in a UCP2 dependent manner.** (**A**) UCP2 protein in WT and AKO macrophage cell lines (macrophage cells lines are denoted by $WT_{MΦ}$ and $AKO_{MΦ}$). (**B**) Hydrogen peroxide levels measured in WT macrophages with or without 4 hr treatment with 30 μM HTS01037 compared to that in AKO macrophages. (**C**) Hydrogen peroxide levels in WT and AKO macrophages treated with or without 100 ng/mL LPS for 24 hours. (**D**) Hydrogen peroxide in WT and AKO control cells (WT-$eGFP_{MΦ}$, AKO-$eGFP_{MΦ}$) and in UCP2 silenced AKO cells (AKO-$UKD_{MΦ}$). (**E**) Hydrogen peroxide measured in Raw 264.7 control cells (Raw-eGFP) and UCP2 silenced Raw 264.7 cells (Raw-UKD) with or without treatment of 30 μM HTS01037 for 16 hours. (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$)

Since the redox environment is different between cultured WT and AKO macrophages, the redox status of cysteine residues in different fractions of the visceral adipose tissue (VAT) of experimental animals was measured using the biotin-switch assay. As shown in Figure 2A, when WT mice were maintained on a high saturated fat diet for 12 weeks, the level of mitochondrial cysteine oxidation in the VAT was markedly greater than in mice maintained on a low fat, chow control diet. When such modification was evaluated in WT mice and compared to FABP4/aP2 null animals (maintained on a high saturated fat diet), mitochondrial cysteine oxidation levels were significantly blunted in the AKO mice, even though these mice were as obese as the wild type controls (Figure 2B). Moreover, the proteins from the whole cell lysates of the VAT derived stromal vascular fraction (SVF) enriched for macrophages, also exhibited a reduction in cysteine oxidation from AKO mice compared to the control animals ($WT_{SVF}$ and $AKO_{SVF}$, Figure 2C). Finally, when the macrophage cell lines were treated with 100 ng/mL LPS for 24 hours, there was less cysteine oxidation in the whole cell lysate of AKO macrophages, albeit to a lesser degree as observed in the tissue samples (Figure 2D). In sum, these results indicate the oxidation of mitochondrial cysteine residues are significantly increased due to a high fat diet in wild type mice, but this is protected against in mice lacking FABP4/aP2.


A
kDa
WT Chow
WT HFD
100
75
50
37
25
20
Cysteine Oxidation
ATP5α
B
kDa
WT HFD
AKO HFD
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75
50
37
25
20
15
Cysteine Oxidation
ATP5α
C
kDa
WTSVF
AKOSVF
100
75
50
37
25
20
Cysteine Oxidation
β-actin
D
kDa
WTMϕ LPS
AKOMϕ LPS
100
75
50
37
25
20
15
Cysteine Oxidation
β-actin

**Figure 2: Loss of FABP4/aP2 in macrophages decreases oxidative modification of cysteine residues.** (**A**) Cysteine oxidation in the mitochondrial fraction of visceral adipose tissue from C57Bl/6J mice on a chow or high saturated fat diet. **(B)** Cysteine oxidation in the mitochondrial fraction of visceral adipose tissue from WT and AKO C57Bl/6J mice maintained on a high saturated fat diet. (**C**) Cysteine oxidation in the whole cell lysate of the stromal vascular fraction of the visceral adipose tissue from wild type and FABP4/aP2 null mice ($WT_{SVF}$ and $AKO_{SVF}$). (**D**) Cysteine oxidation was measured in whole cell lysates of WT and AKO macrophage cell lines following a 24 hour treatment with 100 ng/mL LPS. The intensity of the entire lane for each sample was normalized to internal controls ATP5$\alpha$ or $\beta$-actin to determine changes in cysteine oxidation, and the difference in all experiments were significant by at least $p < 0.05$. Spacing between the blot images in panel C indicates samples from the same membrane and exposure but rearranged for presentation purposes.

**Loss of FABP4/aP2 in the SVF of high fat fed mice leads to upregulation of antioxidants and 20S proteasome subunits.** Changes in redox status of cells is frequently associated with correlative changes in expression of proteins linked to redox biology, particularly anti-oxidants and proteins involved in quality-control systems (9, 33-35). To address this, we evaluated several antioxidants in visceral adipose derived SVF in high fat fed WT and AKO mice. While transcript levels of catalase and superoxide dismutase 2 (*Sod2*) were not significantly different between WT and AKO cells (Figure 3A), there was a significant increase in the protein expression for both antioxidants in AKO animals (Figure 3B-C). Furthermore, the 20S proteasome, which is responsible for recognizing and turning over oxidized proteins in an ATP-independent manner, was also upregulated in both AKO SVF and the AKO macrophage cell line (Figure 3B-D). Finally, in addition to the protection observed in cysteine oxidation, the SVF from AKO mice maintained on a high saturated fat diet revealed an upregulation in methionine sulfoxide reductase A expression (Figure 3E). These results demonstrate that loss of FABP4/aP2 reprograms the redox biology of macrophage cells towards a more reduced environment through two processes; reduced hydrogen peroxide production and upregulation of antioxidants and quality control systems.

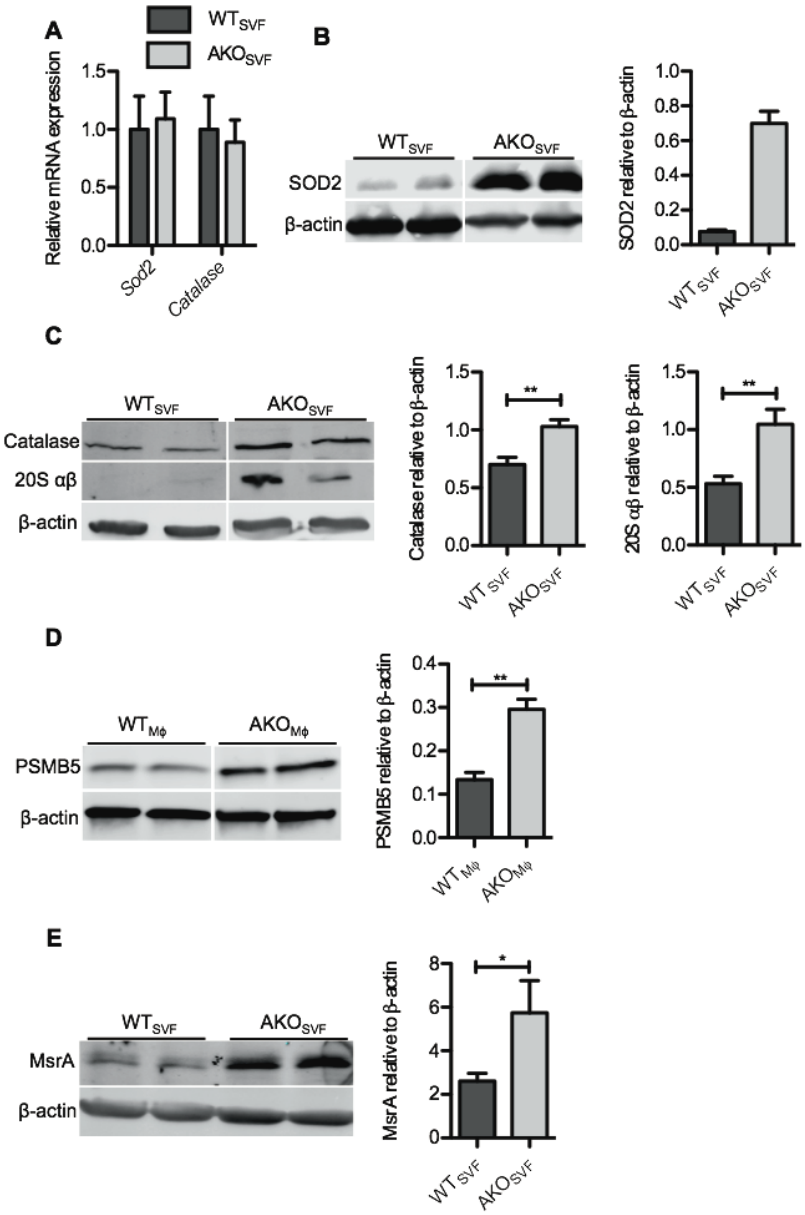

A
WT SVF
AKO SVF
Relative mRNA expression
1.5
1.0
0.5
0.0
Sod2
Catalase
B
WT SVF
AKO SVF
SOD2
β-actin
SOD2 relative to β-actin
1.0
0.8
0.6
0.4
0.2
0.0
WT SVF
AKO SVF
C
WT SVF
AKO SVF
Catalase
20S αβ
β-actin
Catalase relative to β-actin
1.5
1.0
0.5
0.0
**
WT SVF
AKO SVF
20S αβ relative to β-actin
1.5
1.0
0.5
0.0
**
WT SVF
AKO SVF
D
WT Mφ
AKO Mφ
PSMB5
β-actin
PSMB5 relative to β-actin
0.4
0.3
0.2
0.1
0.0
**
WT Mφ
AKO Mφ
E
WT SVF
AKO SVF
MsrA
β-actin
MsrA relative to β-actin
8
6
4
2
0
*
WT SVF
AKO SVF

**Figure 3: Expression of antioxidants and the 20S proteasome in stromal vascular cells of FABP4/aP2 null macrophages.** The SVF of visceral adipose tissue from high fat fed WT and AKO mice were analyzed for (**A**) manganese Sod2 and catalase mRNA, (**B**) protein of manganese SOD2, (**C**) catalase and the $\alpha\beta$ subunit of the 20S proteasome. **(D)** The AKO and wild type macrophage cell lines were analyzed for the expression of PSMB5, the catalytic subunit of the 20S proteasome. (**E**) Methionine sulfoxide reductase (MsrA) levels in the SVF of WT and AKO high fat fed mice. Spacing between the western blot images indicates samples from the same membrane and exposure but rearranged for presentation purposes. (*$p < 0.05$, **$p < 0.005$)

**FABP4/aP2 regulates activation of the mitochondrial unfolded protein response.** One of the consequences of a heightened cellular oxidation and concomitant protein modification is activation of the unfolded protein response (15, 36, 37). Indeed, oxidative modification of mitochondrial proteins often leads to misfolded or aggregated proteins and the increased expression of a cluster of proteases (ClpP and LonP1) designated for proteolysis and clearance of aberrant polypeptides (37). To test this in the FABP4/aP2 model system, mitochondrial unfolded protein response (mtUPR) markers were evaluated in the adipose derived stromal vascular fraction isolated from high fat diet fed wild type and FABP4/aP2 null mice. Consistent with the differences in mitochondrial oxidation status shown in Figure 2, expression of the mtUPR markers in the AKO derived stromal vascular fraction were almost completely ablated compared to that from wild type mice (Figure 4A). Extending this observation from the animal models to cell lines, the AKO macrophage cell line also had a significantly reduced mtUPR as measured by ClpP and LonP1 (Figure 4B). This protection was also shown to be dependent on UCP2 expression as silencing of UCP2 in the AKO macrophage cell line led to an increased expression of LonP1, ClpP and Hsp60 (Figure 4C). Similar to the results in the SVF fraction and AKO cell line, ClpP and LonP1 are also significantly decreased in bone marrow derived macrophages (BMDMs) isolated from FABP4/aP2 null mice compared to wild type littermate controls (Figure 4D). These results indicate that loss of FABP4/aP2 and concomitant change in redox environment attenuates the oxidation of mitochondrial proteins and the up regulation of the mitochondrial unfolded protein

response through expression of UCP2. Consistent with this, Xu et al., have reported that mitochondrial function as measured by cellular respiration and responsiveness to LPS challenge is improved in FABP4/aP2 null macrophages compared to mitochondria from wild type cells (3).

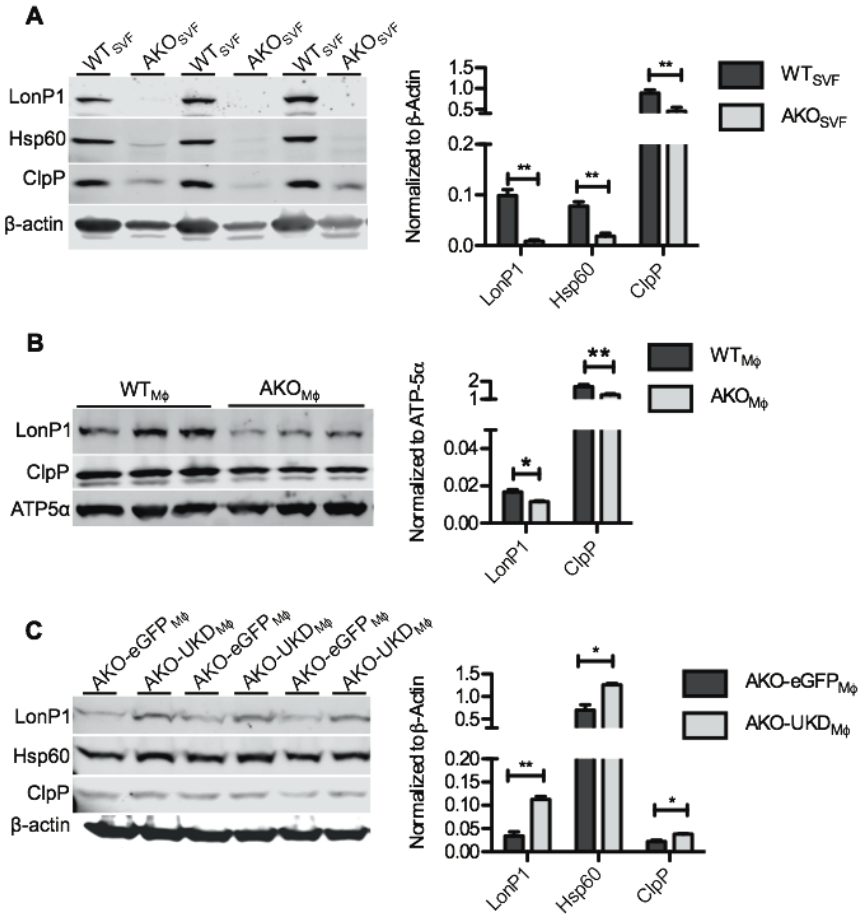



**Figure 4: Expression of LonP1, Hsp60 and ClpP in stromal vascular cells, BMDMs and macrophage cell lines from FABP4/aP2 null and control mice.** (**A**) LonP1, Hsp60 and ClpP in stromal vascular cells from visceral adipose tissue of high fat fed WT and AKO mice. (**B**) LonP1 and ClpP in WT and AKO macrophage cells lines. (**C**) LonP1, Hsp60 and ClpP in AKO-eGFP (AKO-$eGFP_{M\Phi}$) control and AKO-UCP2 knockdown (AKO-$UKD_{M\Phi}$) macrophages. (**D**) Bone marrow derived macrophages from WT and AKO mice were treated with 100 ng/mL LPS for 24 hours and LonP1 and ClpP protein levels were measured (*$p < 0.05$, **$p < 0.005$).

**Maintaining mitochondrial homeostasis through deletion or inhibition of FABP4/aP2 prevents inflammasome activation and IL-1β secretion.**

Reducing ROS levels blunt the NF-κB pathway and stabilizes the NF-κB inhibitory subunit, IκB-α (38, 39). As shown in Figure 1, hydrogen peroxide is significantly reduced in AKO macrophages, prompting us to measure the protein level of IκB-α. As expected, the SVF from AKO mice have significantly more IκB-α compared to WT (Figure 5A). NF-κB regulates the expression of the inflammasome members such as caspase-1, NLRP3 and IL-1β. Consistent with this, caspase-1 protein expression was significantly decreased in AKO macrophage cell lines and AKO BMDMs upon LPS stimulation compared to the respective WT controls (Figure 5B-D). This was also observed when message levels of Nlrp3 were measured in macrophage cell lines and BMDMs. In both cell models, Nlrp3 was significantly decreased when FABP4/aP2 was genetically ablated and when pharmacologically inhibited using HTS01037 in BMDMs prior to LPS stimulation (Figure E-F). This finding was corroborated at the protein level with a significant decrease in NLRP3 protein measured in the AKO cell line compared to the WT control (Figure 5G). NF-κB also regulates the expression of the inflammasome substrate, IL-1β, and upon LPS stimulation, WT macrophages exhibited an increase in IL-1β transcript levels. On the other hand, AKO cells were protected from this increase in IL-1β message, but the protection was lost when UCP2 was silenced (Figure 6A-B). The reduction in IL-1β was also observed in WT BMDMs pre-treated with HTS01037 and AKO BMDMs upon LPS treatment (Figure 6C).

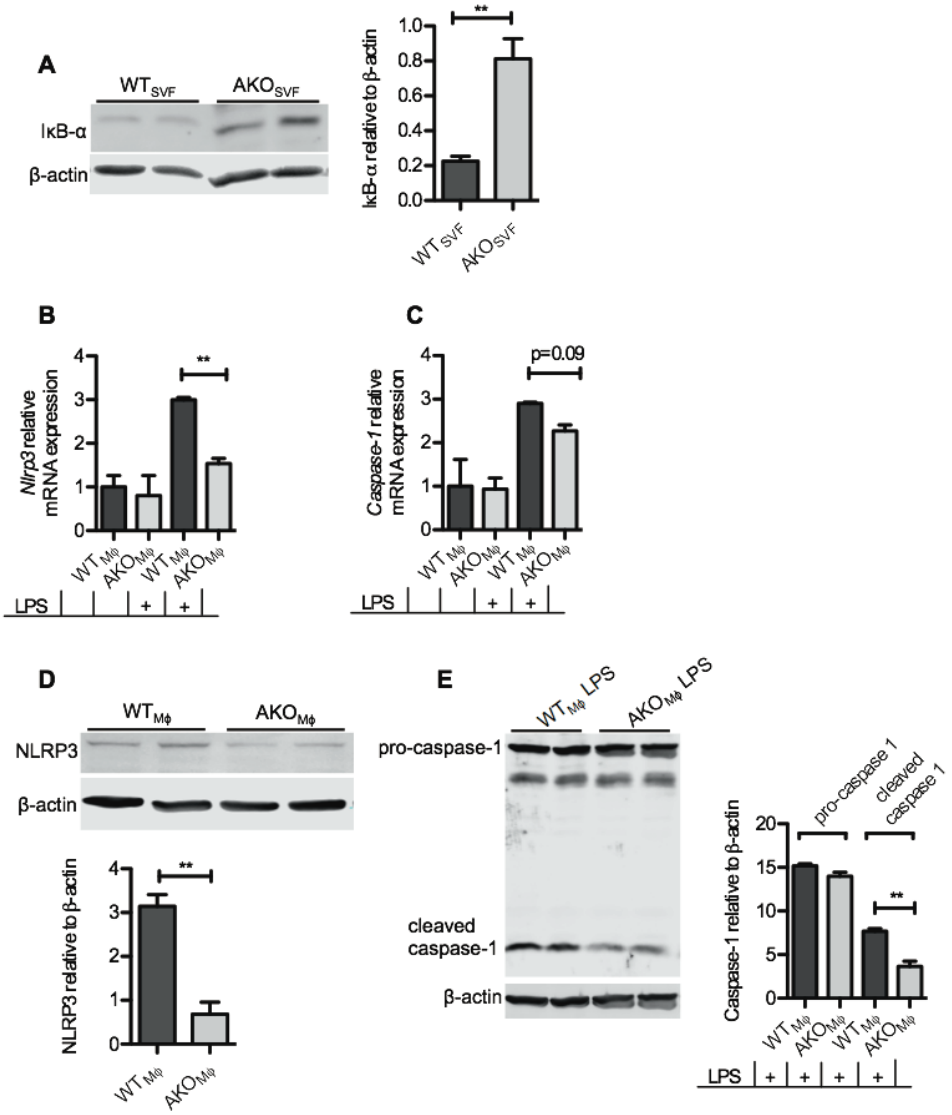

A
WT SVF
AKO SVF
IκB-α
β-actin
IκB-α relative to β-actin
**
WT SVF
AKO SVF
B
Nlrp3 relative mRNA expression
**
WT Mϕ
AKO Mϕ
WT Mϕ
AKO Mϕ
LPS
+
+
C
Caspase-1 relative mRNA expression
p=0.09
WT Mϕ
AKO Mϕ
WT Mϕ
AKO Mϕ
LPS
+
+
D
WT Mϕ
AKO Mϕ
NLRP3
β-actin
NLRP3 relative to β-actin
**
WT Mϕ
AKO Mϕ
E
WT Mϕ LPS
AKO Mϕ LPS
pro-caspase-1
cleaved caspase-1
β-actin
pro-caspase 1
cleaved caspase 1
Caspase-1 relative to β-actin
**
WT Mϕ
AKO Mϕ
WT Mϕ
AKO Mϕ
LPS
+
+
+
+

**Figure 5: Genetic ablation of FABP4/aP2 up regulates IκB-α and reduces caspase-1 and NLRP3 expression.** (**A**) Expression of IκB-α in stromal vascular cells of visceral adipose from high fat fed WT and AKO mice. **(B)** Caspase-1 mRNA in WT and AKO macrophage cells treated with or without 100 ng/mL LPS for 24 hours. (**C-D**) Caspase-1 protein expression in WT and AKO macrophage cell lines and BMDMs following 100 ng/mL LPS for 24 hours. (**E**) Nlrp3 mRNA in WT and AKO macrophages with or without 100 ng/mL LPS for 24 hours and (**F**) measured in WT and AKO BMDMs treated with +/- 30 uM HTS01037 for 4 hours followed by +/- 500 ng/mL LPS for 4h hours. (**G**) NLRP3 protein expression in WT and AKO macrophages. (*$p < 0.05$, **$p < 0.005$)

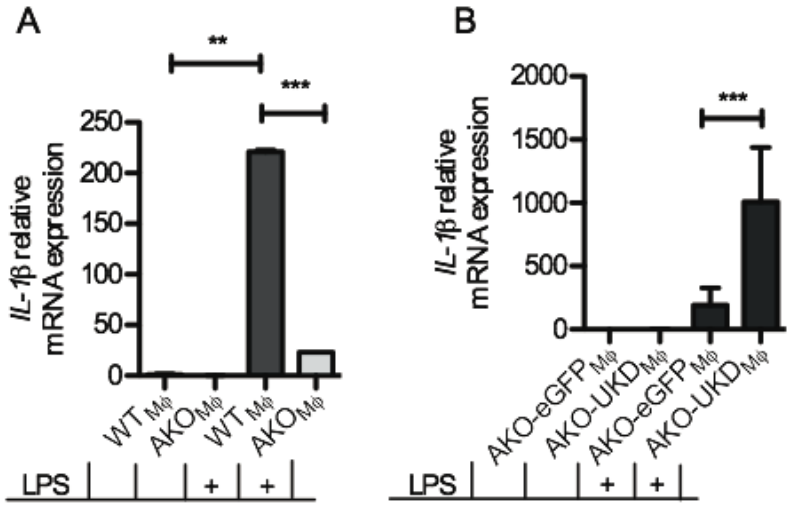
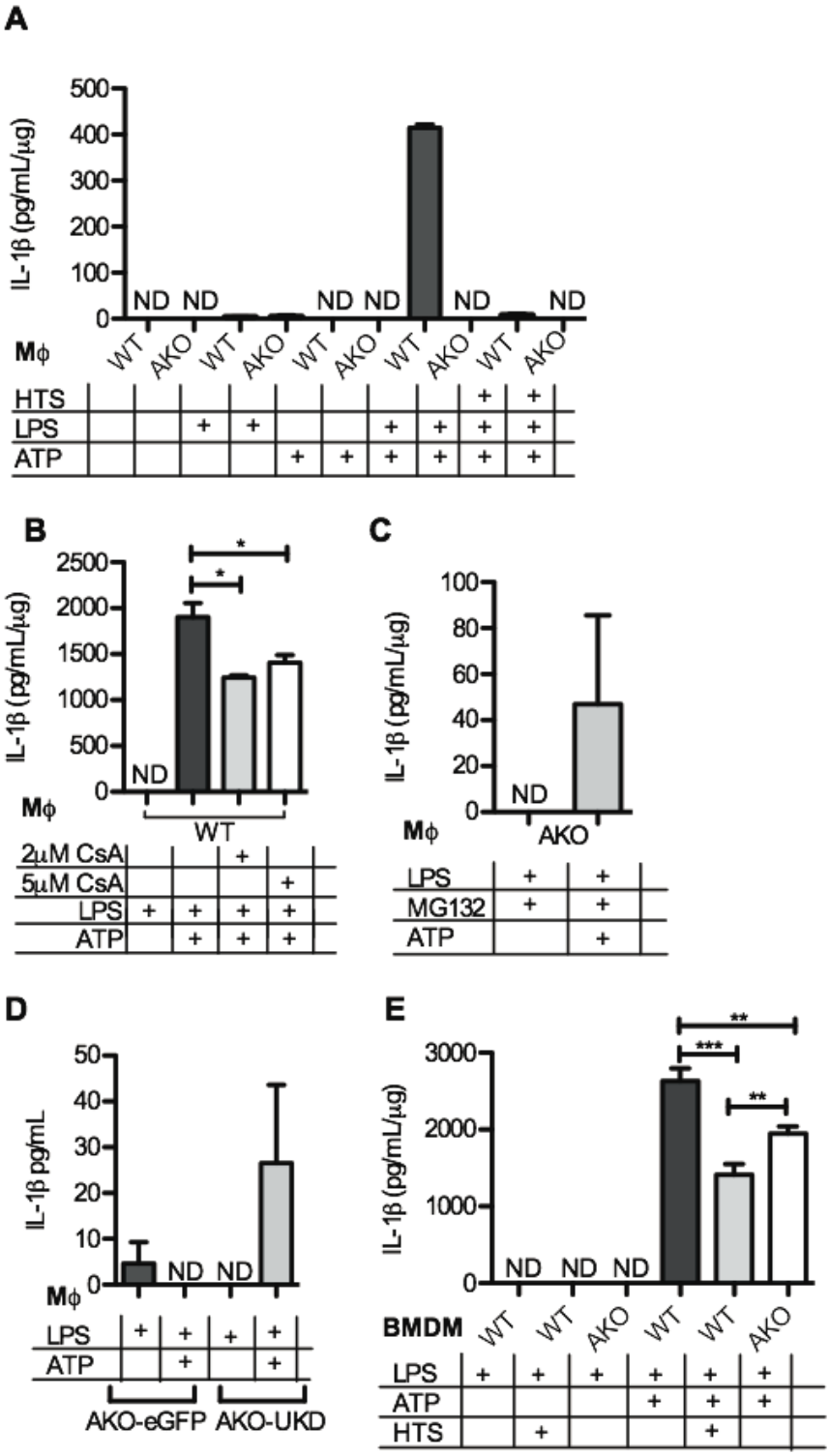



**Figure 6: Decrease in transcript levels of IL-1β in AKO macrophages is dependent on expression of UCP2.** (**A**) IL-1β mRNA measured in WT and AKO macrophages with or without 100 ng/mL LPS for 24 hours. (**B**) IL-1β mRNA measured in AKO-eGFP control and AKO-UKD macrophages in the same conditions as (**A**) (**p < 0.005, ***p < 0.0005).

The bioactive form of IL-1β must be generated by inflammasome-dependent cleavage and secretion, which can be triggered by ROS (as well as other sources) through the mitochondrial intrinsic apoptotic pathway (22, 40). To assess activation of the inflammasome in the FABP4/aP2 system, IL-1β secretion was evaluated in response to either pharmacologic or genetic ablation of FABP4/aP2. Secretion of IL-1β was completely absent in FABP4/aP2 null macrophages and wild type macrophages treated with HTS01037 compared to wild type controls when treated with LPS and ATP (Figure 7A). Furthermore, inflammasome activation was shown to be partially activated by the mitochondrial intrinsic apoptotic pathway, as observed when WT macrophage cells were treated with cyclosporine A (CsA) in addition to LPS and ATP stimulation. As shown in Figure 7B, CsA could blunt the secretion of IL-1β. Conversely, IL-1β secretion could be induced in AKO macrophages by inhibiting the proteasome with MG132 or by silencing UCP2 (Figure 7C-D), indicating that AKO cells maintain some capacity for inflammasome activation through UCP2 expression and protein unfolding. Both mitochondrial-induced apoptosis and protein unfolding have been shown to activate the inflammasome and may explain the upregulation of the 20S proteasome observed in AKO cells (21, 22, 41, 42). Consistent with the immortalized cell lines, bone marrow derived macrophages from AKO or WT mice pre-treated with HTS01037 exhibited a significant decrease in IL-1β secretion upon stimulation with LPS and ATP (Figure 7E).


A
IL-1β (pg/mL/μg)
500
400
300
200
100
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ND ND ND ND ND ND
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WT AKO WT AKO WT AKO WT AKO WT AKO
HTS + +
LPS + + + + + +
ATP + + + + + +
B
IL-1β (pg/mL/μg)
2500
2000
1500
1000
500
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ND
Mϕ
WT
2μM CsA +
5μM CsA +
LPS + + + +
ATP + + +
C
IL-1β (pg/mL/μg)
100
80
60
40
20
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ND
Mϕ
AKO
LPS + +
MG132 + +
ATP +
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IL-1β pg/mL
50
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ND ND
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LPS + + + +
ATP + +
AKO-eGFP AKO-UKD
E
IL-1β (pg/mL/μg)
3000
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ND ND ND
BMDM WT WT AKO WT WT AKO
LPS + + + + + +
ATP + + +
HTS + +

**Figure 7: Secretion of IL-1β is reduced under pharmacologic and genetic ablation of FABP4/aP2.** (**A**) IL-1β secretion with or without 4 hour treatment of 30 μM HTS01037 followed by 500 ng/mL LPS for 4 hours followed by 2 mM ATP for one hour. (**B**) IL-1β secretion in WT macrophages in the presence of 2 μM or 5 μM cyclosporine A for 1 hour followed by LPS and ATP treatment as described in (**A**). (**C**) AKO macrophages treated with LPS and ATP alone as in (**A**) or with the addition of 10 μM MG132 for 2 hour after LPS treatment and IL-1β secretion was measured. (**D**) IL-1β secretion measured after LPS treatment with or without ATP as in (**A**) and compared between AKO-eGFP control and AKO-UKD macrophages. (**E**) IL-1β secretion measured in BMDMs from WT and AKO mice (with or without a 4 hour pre-treatment of 30 μM HTS01037) treated for 4 hours with 500 ng/mL LPS followed by 2 mM ATP for one hour. (*$p < 0.05$, **$p < 0.005$)

## DISCUSSION

Loss of the adipocyte fatty acid-binding protein has been shown to be protective against the low-grade chronic inflammation that leads to metabolic dysfunction under obese conditions (24, 27). This is largely due to the phenotypic switch of adipose tissue macrophages from an inflammatory to anti-inflammatory state with concomitant changes in cytokine and chemokine secretion (25, 26). While the major physiological outcomes have been described, the molecular mechanisms that underlie such regulation have remained enigmatic. Herein, we show for the first time the major finding that macrophage redox biology is regulated by the FABP4/aP2-UCP2 axis, resulting in changes in protein oxidation and preventing the induction of the mitochondrial unfolded protein response and inflammasome activation (Figure 8).

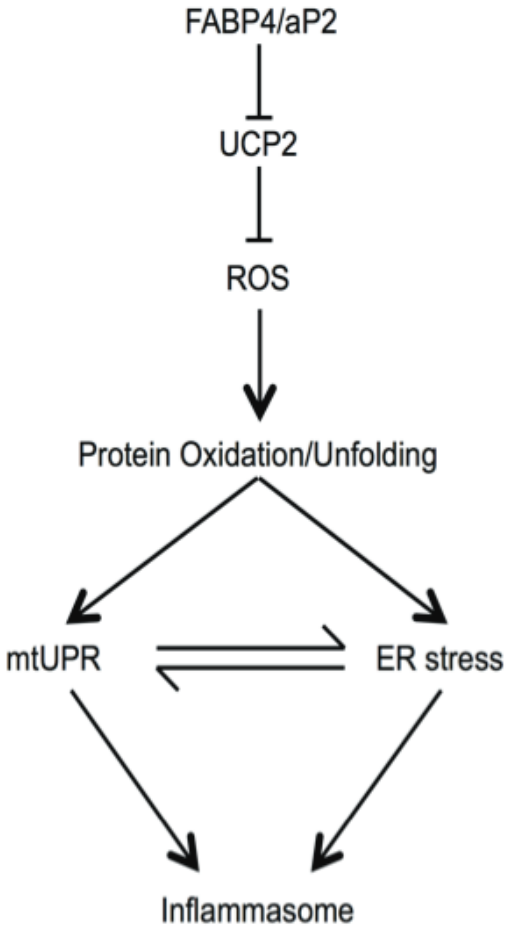


**Figure 8. Schematic representation of the FABP4/aP2-UCP2 axis and subsequent regulation of the inflammasome.** Genetic or pharmacologic inhibition of FABP4/aP2 increases the level of intracellular free fatty acids (e.g. palmitoleic acid). This in turn leads to increased expression and activation of UCP2 that decreases $H_2O_2$ levels. Lowering $H_2O_2$ attenuates protein oxidation and activation of the mtUPR. ER-mitochondrion communication activates the inflammasome and subsequent IL-1$\beta$ secretion.

We have previously shown that loss of FABP4/aP2 leads to an upregulation of UCP2 and Sirt3 in macrophages (3, 28). We now observe that AKO macrophages are protected from LPS-induced hydrogen peroxide production, and UCP2 regulates this process. The difference in hydrogen peroxide ultimately protects AKO cells from hyper-oxidation of cysteine (and likely methionine) residues following 12 weeks of a high fat diet. This was observed in the mitochondria and stromal vascular fractions of VAT isolated from AKO mice compared to WT mice and further confirmed in immortalized macrophage cell lines following LPS stimulation. Interestingly, the difference in cysteine oxidation was less dramatic in the cell lines compared to the tissue samples, potentially due to different signaling nodes being activated under obese conditions as opposed to a single treatment of LPS. Moreover, protein levels of inflammasome complex members and mtUPR markers in Sirt3 silenced macrophage cells are not different compared to the wild type cells. This suggests that changes in the mtUPR and inflammasome activation are likely to be independent from Sirt3 but are downstream of UCP2 (results not shown).

Because UCP2 is a mitochondrial protein that reduces oxidative stress and mitochondrial protein oxidation, we focused on downstream effects potentially mediated by the mitochondrial unfolded protein response. Indeed, LonP1, ClpP and Hsp60 were markedly reduced both in the AKO SVF, BMDMs and macrophage cell lines from AKO mice when compared to controls. This protection was also shown to be dependent on the expression of UCP2 as the

mitochondrial proteases were increased in UCP2 silenced cells compared to eGFP controls in the AKO background. It is also of interest to note that the most robust changes observed in these different cell lines consistently came from LonP1, which specifically recognizes and targets oxidized proteins for degradation (37). Compromising homeostasis of the mitochondrion can lead to apoptosis, and one of the primary downstream effectors of the mtUPR in immune cells is the activation on the inflammasome (22, 40, 43, 44). This was prevented in cells lacking FABP4/aP2 as observed by the decrease in mRNA and protein levels of the inflammasome members as well as its activation (IL-1β secretion). The activation of the inflammasome in WT macrophages could be partially rescued with CsA, inhibitor of the mitochondrial intrinsic apoptosis pathway, and conversely, IL-1β secretion could be induced in AKO cells by inhibiting the proteasome or by silencing UCP2 (22). This indicates that both mitochondrial integrity and protein folding, potentially through redox status, are maintained through the FABP4/aP2-UCP2 axis. Furthermore, this system was also tested in BMDMs, and while there was a detectable amount of IL-1β from the AKO and WT HTS01037 treated cells, both conditions exhibited a significant decrease compared to WT BMDMs following LPS and ATP stimulation (Figure 7).

ER-mitochondrial communication is a dynamic process driven by dedicated structural domains within mitochondria-associated membranes ((45-47). Previous work by Xu et al. (3) has shown that ER stress is blunted in FABP4/aP2 ablated macrophage cells due to increased expression of UCP2. Paralleling this,

Bronner et al. demonstrated that ER stress leads to mitochondrial ROS production which in turn promotes inflammasome dependent IL-1β secretion (16). These observations suggest that in macrophages, inflammasome activation may be potentiated by either ER stress or the mtUPR and that organelle communication may facilitate signaling. The FABP4/aP2-UCP2 axis is upstream of these events and implicates redox balance as being pivotal in initiating the inflammatory response (Figure 8).

The major regulator of IL-1β expression is mediated through the NF-κB pathway and its activation is up regulated through hydrogen peroxide signaling (48, 49). Indeed, hydrogen peroxide signaling is essential for full activation of inflammatory macrophages, and blunting such processes inhibits NF-κB activation (38, 48, 50, 51). Consistent with this, Figure 5 shows that IκB-α, the major inhibitor of NF-κB, was upregulated in the SVF of AKO mice. The mechanism for this up regulation is unresolved but may be due to enhanced stability from a less oxidizing environment (38, 39). In support of IκB-α upregulation in AKO SVF, NF-κB targets, NLRP3 and caspase-1 were decreased in AKO macrophages. IL-1β message levels were also decreased in AKO macrophages, and in agreement with others studies, was shown to be dependent on UCP2 expression (32). Another transcription factor that may be playing a role in altered redox status of FABP4/aP2 null cells is nuclear factor (erythroid-derived 2)-like 2 (Nrf-2). It is the master regulator of antioxidants, such as SOD2 and catalase as well as the 20S proteasome and UCP2 (42, 52, 53). While the antioxidants and UCP2 are able to

decrease hydrogen peroxide, the 20S proteasome is uniquely able to recognize and degrade oxidized proteins to prevent subsequent unfolding (41). Determining the localization and activation of both NF-κB and Nrf-2 will be an essential next step in understanding the role of the FABP4/aP2 – UCP2 axis in controlling inflammasome activation in response to obesity.


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# CHAPTER FIVE

## Differential Irg1 expression pattern in LPS treated macrophages

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This chapter contains unpublished work

Kaylee Steen wrote and generated the data (with the exception of figure 3A)

## SUMMARY

The study of metabolic flux changes that occur in response to cellular need has been an exciting area of research over the past several years. Macrophages are no exception to this, and metabolic alterations have shown to be an obligatory process in order to carry out robust inflammatory responses. The mitochondria are a particular hot spot of metabolic regulation as reactive oxygen species and other key signaling metabolites accumulate here. Itaconate is one such metabolite that is produced after an inflammatory stimulus in order to reestablish homeostasis, and we have demonstrated this process is regulated by the expression of the fatty acid-binding protein 4 (FABP4). While basal expression of Irg1 and subsequent production of itaconate are lower in FABP4 knockout macrophages compared to wild-type cells, there is a robust increase in Irg1 levels upon longer treatment of LPS. These data indicate that while FABP4 null macrophages are initially less inflammatory and thus require less itaconate, these cells have an enhanced compensatory mechanism in which to resolve inflammation.

## INTRODUCTION

Macrophages are extremely efficient in their ability to respond to local signals in their environment. One such way this is accomplished is through altering metabolic pathways that produce necessary metabolites for rapid proliferation, production of reactive oxygen species (ROS) or inflammatory resolving metabolites. This can be accomplished in a variety of ways, such as transcriptional control of glycolytic enzymes by HIF-1$\alpha$ that increases glycolytic flux in response to inflammatory stimuli (1). Macrophages also alter metabolic metabolite levels by regulating mitochondrial dynamics through fission and fusion events. During fission, the complexes in the electron transport chain become disordered and less compact, increasing the movement of electrons into the mitochondrial matrix and ultimately increasing ROS levels (2). This heightened oxidative state is required in order to defend against pathogens, but macrophages must be able to resolve this inflammation once the infection has been cleared. Under resolving conditions, the mitochondria undergo a greater rate of fusion events that reorganize the electron transport chain. This allows the cell to rely on oxidative phosphorylation instead of glycolysis, reducing ROS levels and proliferation.

Another mechanism in which to dampen an inflammatory response is to accumulate the metabolite itaconate, which is derived from cis-aconitate in the tricarboxylic acid (TCA) cycle by the immune responsive gene 1 (Irg1). Transcriptional and metabolomics profiling of bone marrow derived macrophages

(BMDMs) under basal and lipopolysaccharide (LPS) conditions showed that Irg1 expression and itaconate production were two of the most robust changes following inflammatory stimulus (3). Based on these data, it was initially hypothesized that itaconate was an essential inflammatory marker of an M1 macrophage. However, itaconate is also known to have antimicrobial affects and under further investigation, itaconate production was demonstrated to be required for the reduction in IL-1β and IL-18 cytokine secretion in BMDMs (4).

The expression of the fatty acid-binding protein 4 (FABP4) has demonstrated to be a positive regulator of macrophage inflammation and subsequent metabolic disease. This inflammation can be prevented through genetic knockout or pharmacologic inhibition of FABP4 but is lost when the uncoupling protein-2 is silenced (5, 6). This led us to hypothesize FABP4 modulates the expression of Irg1. In order to test this, FABP4 wild type (WT) and knockout (AKO) macrophage cell lines were treated under basal and LPS stimulated conditions and Irg1 expression was examined.

## MATERIALS AND METHODS

**Mice.** Male WT and AKO C57BL/6 mice were maintained on a chow diet (BioServe) and fed *ad libitum*. The hind legs were harvested for bone marrow isolation between 8-10 weeks of age. All experimental procedures using animals were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee

**Cell culture.** Macrophage FABP4/aP2 knockout (AKO), wild type and UCP2 knockdown in an AKO background were maintained in RPMI 1640 (Invitrogen) with 5% fetal bovine serum(5). The macrophages were immortalized from bone marrow derived macrophages of chow fed WT and AKO C57BL/6 mice(7). Raw264.7 macrophages and UCP2 knockdown Raw264.7 macrophages were maintained in DMEM (Invitrogen) with 10% fetal bovine serum (FBS)(5). Bone marrow derived macrophages were isolated from WT and AKO C57BL/6 mice between 8-10 weeks of age and maintained in Iscoves's Modified Dulbecco's Medium (Invitrogen), 10% FBS and 10ng/mL macrophage colony-stimulating factor (M-CSF) for one week. $2X10^6$ cells were plated in 6-well plates prior to LPS stimulation(8).

**Quantitative RT-PCR**. Total RNA was isolated using TRIzol reagent (Invitrogen) from WT, AKO and AKO-UKD macrophage cell lines. cDNA synthesis was performed by using iScript according to the manufacturer's protocol (Bio-Rad). qRT-PCR amplification utilized a Bio-Rad CFX 96 real-time system with a SYBR green Supermix (Bio-Rad). TATA-binding protein (TBP) was used as an internal control to normalize expression. Primer sequence for IL-6 are forward: CAAGAGATACAAAGAAATGATGG and reverse: ACTCCAGAAGACCAGAGGAAAT

**Immunoblot analysis.** Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with fresh protease inhibitors. All samples were

normalized to protein concentration prior to loading using a bicinchoninic acid (BCA) assay. Between 20-50μg of protein was added for each sample and run on an SDS-PAGE gel followed by transferring to a polyvinylidene difluoride (PVDF) membrane. After blocking with Odyssey blocking buffer (LI-COR Biosciences), membranes were incubated with primary antibody overnight at 4° C. Membranes were washed and incubated with secondary antibody conjugated to Li-Cor IRDye for 1 hr and visualized using Odyssey infrared imaging (Li-Cor Biosciences). The primary antibodies used were anti-Irg-1 (Abcam) and anti-β-actin (Sigma-Aldrich).

**Statistical Analysis.** All statistics were determined as a result of the standard deviation following a two-tailed un-paired student t-test. Experiments were carried out in at least three replicates.

## RESULTS

### Differential expression levels of Irg1 transcript and protein levels.

As shown in figure 1A, transcript levels of Irg1 are significantly reduced in AKO macrophages under basal conditions. However, following 18 hours of LPS stimulation, Irg1 expression in AKO macrophages surpasses that of WT macrophages. We next examined Irg1 expression in BMDMs following a 4-hour LPS treatment. Interestingly, the AKO Irg1 expression had a decreasing trend compared to WT BMDMs (figure 1B). Furthermore, silencing of UCP2 in AKO macrophages (AKO-UKD) increased Irg1 expression under basal conditions, but

that upregulation was lost following longer time course treatments of LPS for 8 and 24 hours (figure 1C).

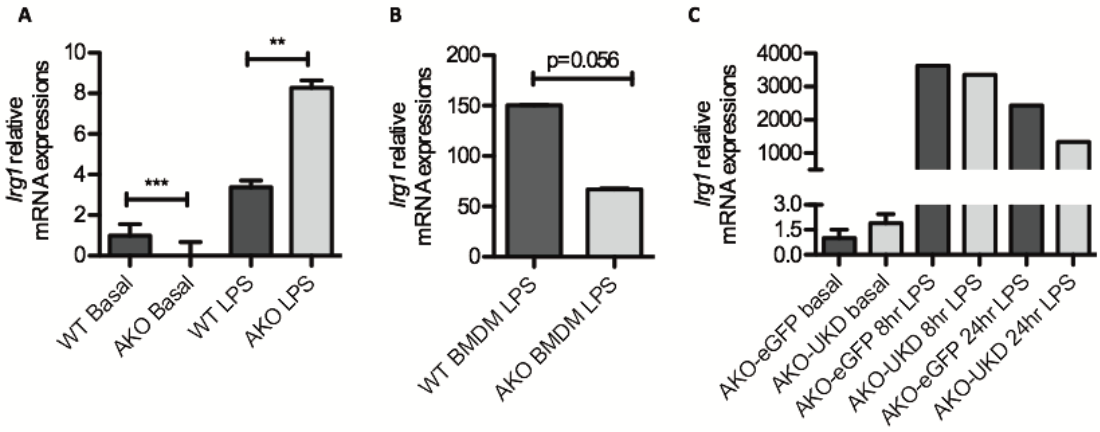


**Figure 1: Differential expression of Irg1 transcript levels in WT and AKO macrophages.** (A) Irg1 transcript levels were measured in WT and AKO macrophages under basal conditions or following 18 hours 100ng/mL LPS treatment. (B) WT and AKO BMDMs were treated with 500ng/mL LPS for 4 hours and Irg1 levels were measured. (C) AKO-eGFP and AKO-UKD macrophage cell lines were treated with 100ng/mL LPS for the indicated time. All experiments were done in replicates of 4 and statistical significance was measured using student's t. test with a *p<0.05, **p<0.005, ***p<0.0005

Due to this interesting observation of a time-dependent expression pattern of Irg1, WT and AKO macrophages were treated with LPS for 0-12 hours and protein levels of Irg1 were assessed. Similarly to the transcript levels, Irg1 protein expression was significantly decreased in AKO macrophages under basal conditions and up to 4 hours of LPS treatments. However, at 7 hours of LPS treatment Irg1 expression was not significantly different between the two cell lines, and AKO Irg1 expression ultimately surpassed WT Irg1 at 12 hours LPS treatment (Figure 2A). To further determine the affects of acute LPS treatment, the small molecular inhibitor of FABP4 (HTS01037) was tested. Pharmacologic inhibition recapitulated the AKO phenotype as Irg1 expression was significantly reduced when pretreated to WT macrophages followed by a 4 hour LPS treatment, but was unchanged in the absence of LPS. This observation in the short LPS treatment was further confirmed in BMDMs (Figures 2B-2C). Finally, UCP2 was silenced in Raw264.7 macrophages and Irg1 expression was measured. Interestingly, there was no difference between control cells (Raw-eGFP) and UCP2 silenced cells (Raw-UKD) under basal conditions, but Irg1 expression could not be induced following 18 hours of LPS stimulus in UCP2 silenced macrophages (Figure 2D). This indicates the enhanced induction of Irg1 in AKO macrophages following a longer treatment of LPS is partially dependent on the presence of UCP2.

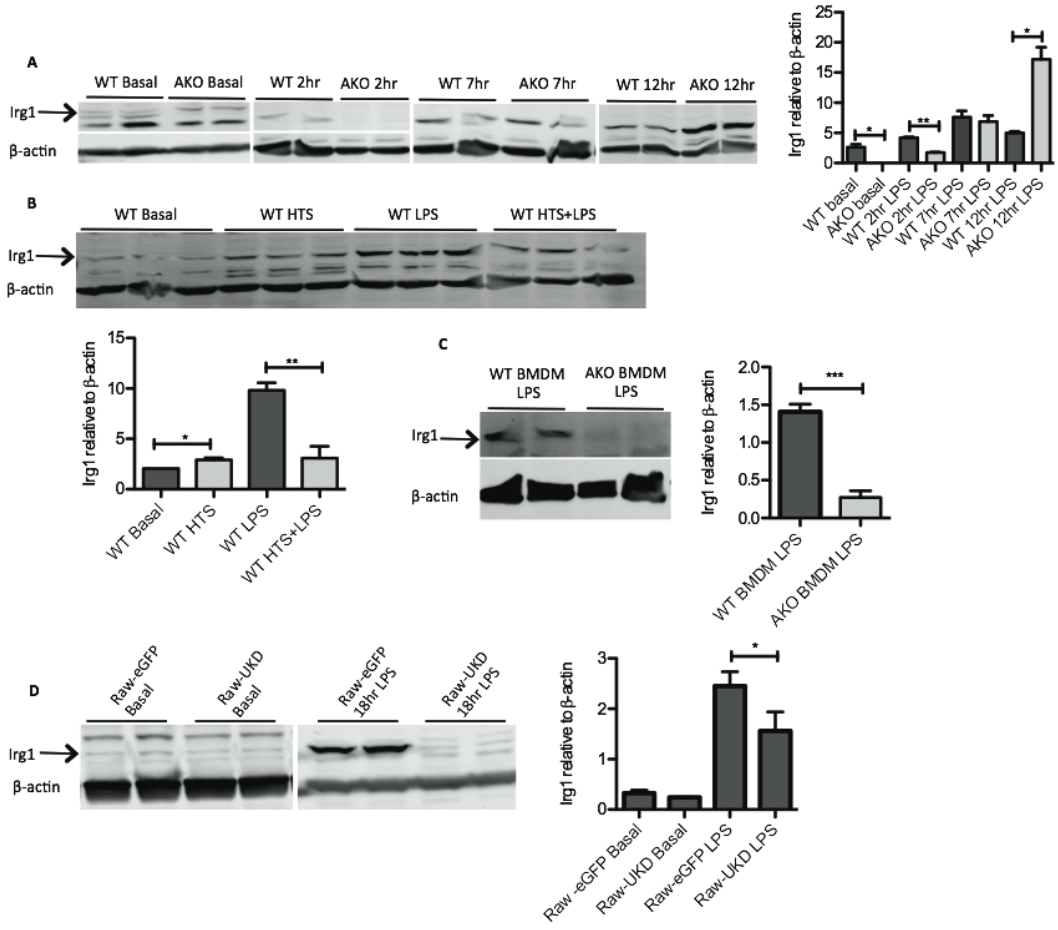


**Figure 2: LPS stimulated Irg1 protein expression follows a time dependent pattern dependent of FABP4 and UCP2 expression.** (A) Time course of 100ng/mL LPS treatment was done in WT and AKO macrophages and Irg1 protein was quantified. (B) WT macrophages were treated with 30uM HTS01037 or 100ng/mL LPS alone for 4 hours each or combined with 4 hours HTS pretreatment followed by 4 hours LPS treatment. Irg1 expression was quantified following cell harvesting (C) WT and AKO BMDMs were treated with 500ng/mL LPS for 4 hours. (D) Stable cell lines Raw264.7 macrophages with either e-GFP (Raw-eGFP) control vector or UCP2 targeted shRNA (Raw-UKD) were developed and Irg1 expression was quantified under basal and 18 hours 100ng/mL LPS treatment. All experiments were done in replicates of 3 or more and statistical significance was measured using student's t. test with a *p<0.05, **p<0.005, ***p<0.0005

**Itaconate production and itaconate inflammatory regulation in WT and AKO macrophages.**

Finally, intracellular itaconate levels were measured in WT, AKO and AKO-UKD macrophages. Following the protein expression patterns of Irg1, itaconate metabolite concentrations were unchanged when WT macrophages were treated with HTS but were significantly reduced in AKO macrophages under basal conditions. There was also a small but significant increase in itaconate concentrations when UCP2 was silenced in an AKO macrophage (Figure 3A). Metabolite levels under different LPS stimuli are still being tested but are predicted to reflect Irg1 protein levels.

With the observation of differing itaconate metabolite levels, we next tested the biological consequence of exogenous itaconate treatment. To do this, the cell permeable dimethyl-itaconate compound was given to WT and AKO macrophages under basal or LPS treated conditions and IL-6 transcript levels were measured. After a 4-hour LPS treatment WT macrophages exhibited a robust increase in IL-6 message levels, which was protected against in AKO macrophages. However, when WT cells were pretreated with 250uM itaconate for 16 hours, IL-6 message levels were dramatically decreased and comparable to IL-6 expression in AKO macrophages (Figure 3B). This indicates the production of itaconate is sufficient in blunting the inflammatory IL-6 response in macrophages and is being regulated by FABP4 activity.

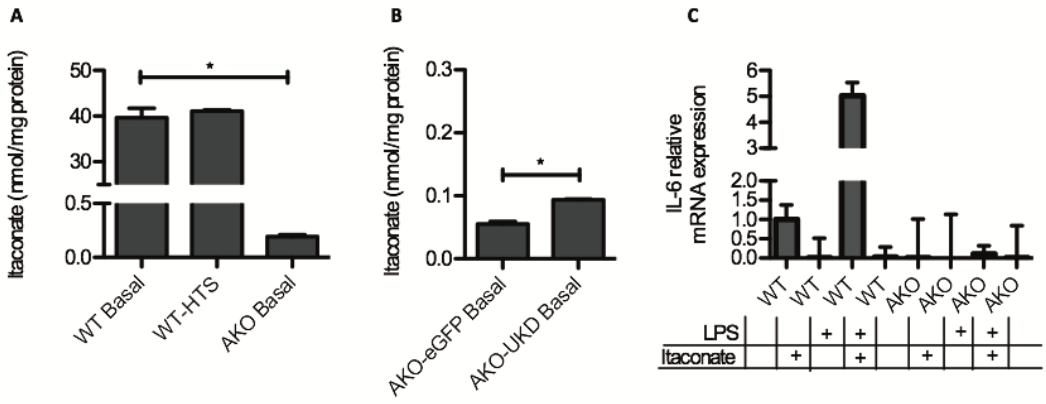


**Figure 3: Itaconate levels are decreased in FABP4 null macrophages and affects inflammation.** (A-B) Cell pellets of WT, AKO, AKO-eGFP and AKO-UKD macrophages were submitted for metabolite analysis of itaconate concentrations under basal conditions. (C) WT and AKO macrophages were treated with 250uM dimethyl-itaconate as indicated for 16 hours followed by treatment of 100ng/mL LPS for 6 hours as indicated. The RNA was isolated from these samples and IL-6 transcript levels were measured. All experiments were done in replicates of 4 and statistical significance was measured using student's t. test with a *$p<0.05$, **$p<0.005$, ***$p<0.0005$

## DISCUSSION

Macrophages and other immune cells alter their metabolic pathways in order for intermediate metabolites to accumulate and help promote an inflammatory response. For instance, upon an inflammatory stimulus, macrophages decrease the rate of oxidative phosphorylation and succinate accumulates as a result. Succinate helps stabilize hypoxia inducible factor-1 alpha (HIF-1$\alpha$), which in turn upregulates various inflammatory cytokines (9). However, while an inflammatory response is necessary to eliminate pathogens, homeostasis must be reestablished. Recently, it has been determined that Irg-1 expression is initiated after an inflammatory response and sequesters cis-aconitate to be metabolized to itaconate. This metabolite has demonstrated to be essential in reducing the inflammatory profile of macrophages (10).

This polarization of macrophages is required for not only immune function in response to infections but also in maintaining the metabolic health in adipose tissue. FABP4 has demonstrated to play a critical role in the inflammatory phenotype in macrophages and loss of FABP4 in macrophages is able to prevent both the low-grade, chronic inflammation and the subsequent metabolic dysfunction (11). Here, we show a connection in the differential expression of Irg1 in WT and AKO macrophages in a time dependent manner following LPS stimulation. While basally, there is less expression of Irg1 and production of itaconate in AKO macrophages, Irg1 expression surpasses that of WT macrophages after longer LPS exposure. Future work is aimed at measuring the

production of itaconate in LPS treated WT and AKO macrophages as well as determine the mechanism in which Irg1 expression is initially decreased in AKO macrophage but robustly increases after longer exposure to inflammatory signals.

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# CHAPTER 6

## CONCLUSIONS AND PERSPECTIVES

Written by Kaylee Steen

Obesity in and of itself is not a disease in the traditional sense. With every body type and genetic background, the body mass index (the measurement in which obesity is defined) will differ between individuals. In fact, not only does the adipose tissue specialize in safely storing excess energy in the form of triglycerides, but the lack of adipose tissue leads to a wide range of syndromes (1-3). Furthermore, the ability to produce more adipocytes (hyperplasia) of a smaller size facilitates both lipid storage and exhibits a secretory profile that maintains metabolic homeostasis (i.e. IL-10 and adiponectin) independent of weight. On the other hand, under obesity-induced metabolic dysfunction, adipocytes typically increase in size and are associated with hypoxic conditions and inflammatory cytokine production that ultimately shifts the immune cell landscape of the tissue (4-6). This inflammatory environment further exacerbates conditions, such as insulin resistance and lipotoxicity as the adipocyte begins to fail in lipid storage capabilities (2).

It is still unclear as to why some individuals develop metabolic disease as a result of obesity while others maintain a relatively normal metabolic profile. While the events described above play a role, the mechanisms in which those fates are determined are not understood. However, what is clear is that by preventing the inflammatory events of the adipose tissue, obesity can be uncoupled from diseases, such as insulin resistance, cardiovascular disorders and hepatosteatosis (7, 8). For this reason, research has gone in to deciphering the secretory factors released by adipocytes that recruit and activate inflammatory

immune cells by potentiating essential signaling events of those immune cells. Many elements have demonstrated to be critical, including the population of T cells, macrophages and Eiosinophils as well as the expression level of various proteins.

The fatty acid-binding protein 4 (FABP4) is one such protein that regulates phenotypic aspects of adipocytes and macrophages. Over the last twenty years, there is no doubt that FABP4 plays a major role in metabolic health, but the mechanism in which this occurs in macrophages is still being determined. This connection is strongly linked to the activation of inflammatory pathways in response to obese conditions and has more recently been associated with the production of reactive oxygen species. In addition to the damaging characteristics of ROS, it has also been shown to be a major signaling system, particularly related to hydrogen peroxide. Through oxidation of cysteine residues, hydrogen peroxide can alter enzymatic activity and protein-protein interactions.

One such avenue this thesis work tried but ultimately was unable to uncover were the proteins that are particularly susceptible to oxidation under high fat diet conditions. As shown in chapter 4, there is a clear increase in global cysteine oxidation when mice are put on a high fat diet for 12 weeks, and that is protected against in the FABP4 knockout mice. In order to fully understand how these oxidizing events alter protein function, stability and interactions, we wanted to identify the proteins and quantify the level of cysteine oxidation in wild type

versus FABP4 null obese mice. This was attempted using an isotope coded affinity tagging technique, but due to the multiple precipitation and solubilizing steps, loss of sample was a major challenge (9). However, there are now labeling techniques that can covalently bind to sulfenic acids, such as dimedone, which has the potential to greatly increase the yield of these experiments and offers the possibility to identify those proteins that are targets for oxidation (10). The greatest challenge with this technique would be to develop differentially tagged dimedone compounds, so samples could be quantifiably compared between different diet and genotype conditions.

Another are of current pursuit following this thesis work is to further elucidate the role of itaconate and the regulatory mechanism in an FABP4 deficient system. Irg1 synthesizes Itaconate in an extremely robust manner following inflammatory stimulation, and it is thought this increased production is to resolve the inflammation and return the macrophage to a homeostatic state. As seen in chapter 5, there is an interesting observation that basally FABP4 knockout macrophages exhibit lower expression levels of Irg1 and production of itaconate compared to wild type macrophages. However, after a longer exposure to LPS (around 12 hours), Irg1 expression in FABP4 null macrophages exceeds wild type macrophages, which still falls in line with the anti-inflammatory nature of an AKO macrophage. Since FABP4 is a fatty acid binding protein, we are currently looking at the affects of fatty acid treatment in these cells. Preliminary results show an increased expression of Irg1 with palmitate treatment and a decreased

expression with palmitoleic acid (data not shown). This differential expression was also dependent on UCP2, but it did not follow the same pattern as the LPS treated macrophages, indicating different signaling pathways between fatty acids and LPS (data not shown). With these complex results, work is ongoing to answer this question as itaconate has recently been shown to be essential in macrophage polarization (11, 12).

In conclusion, the dynamics of cellular organization and signaling is vastly complex in the adipose tissue and is highly sensitive to nutrient conditions. While this work does not tackle the obesity pandemic, it does aim to answer some of the underlying mechanisms in which metabolic syndrome can develop. Inflammation has been shown to be a determinant of not only insulin resistance but also other inflammatory diseases. By understanding the pathways that determine macrophage polarization, there is potential to develop new therapeutics to reduce the low-grade chronic inflammation seen in obese adipose tissue. This would relieve both a great burden on health care costs as well as the cost in quality of life for an individual.

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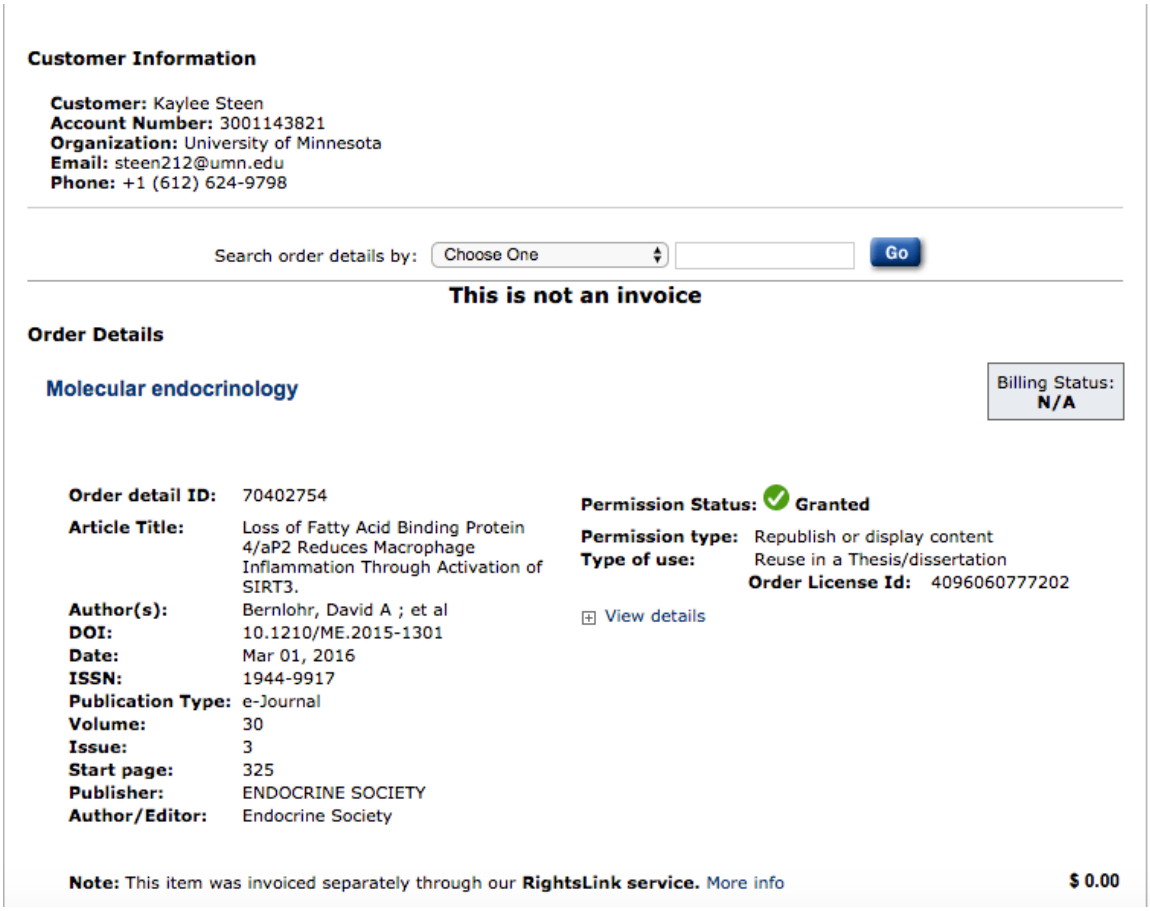

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